MECHANISMS OF ALTERED IMMUNE RESPONSIVENESS IN MICE INFECTED WITH *Trichinella spiralis*

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

AIS	antigen-induced suppression
AFC	antibody-forming cells
BA	bacteria-antibody complex
BAC	bacteria-antibody-complement complex
BSA	bovine serum albumin
Con A	Concanavalin A
cpm	counts per minute
DNP	dinitrophenol
HA	hemagglutinin
HBSS	Hanks' balanced salt solution
Ig	immunoglobulin
LPS	lipopolysaccharide
2-ME	2-mercaptoethanol
PBS	phosphate-buffered saline
PHA	phytohemagglutinin
sem	standard error of the mean
SRBC	sheep erythrocytes

Abstract of Dissertation Presented to the Graduate Council
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Mice infected for 20 days with the parasitic nematode *Trichinella spiralis* had significantly reduced numbers of splenic antibody-forming cells (AFC) and decreased serum hemagglutinin titers following intraperitoneal immunization with sheep erythrocytes. Similarly, when immunized *in vitro* with sheep erythrocytes, cultures of splenocytes from infected mice developed fewer AFC than cultures of normal cells. Splenocytes from infected mice actively suppressed the *in vitro* responses of normal cells to sheep erythrocytes. This *in vitro* suppression was abolished by lysis with anti-thy 1 antiserum and enhanced by lysis with antiimmunoglobulin antiserum, indicating that the suppression was T-lymphocyte dependent. The addition of supernatant fluids from cultures of splenocytes from infected mice to cultures of normal splenocytes on the first day of culture reduced by 70% the number of AFC produced by these cultures. Secretory products and extracts of *Trichinella* larvae also suppressed the AFC responses of normal splenocytes, but sera from

infected mice were no more suppressive than sera from normal mice. Delayed-type hypersensitivity and antibody responses to the T-independent antigen DNP-Ficoll were not suppressed in infected mice. These results indicate the presence of suppressor T-cells in the spleens of infected mice and suggest that antigen-induced suppression might be one important mechanism of Trichinella-induced, spleen-mediated immunosuppression.

Mice infected for 20 days with Trichinella had reduced numbers of AFC in the lymph nodes following subcutaneous immunization with sheep erythrocytes. However, lymph node cells from infected mice developed more AFC than cells from normal mice following in vitro immunization. Although splenocytes from infected mice could suppress in vitro AFC responses of lymph node cells, in vivo suppression did not appear to be dependent on splenic suppressor cells, since splenectomy did not alleviate suppression. Subcutaneous injection of ^{125}I-labelled sheep erythrocytes indicated that the amount of label reaching the draining lymph nodes was the same in normal and infected animals. Lymph nodes of infected mice had an absolute increase in numbers of all cell types, but had a proportionately greater increase in B-cells. This B-cell increase was probably the reason for the increased in vitro responses to sheep erythrocytes, but the reason for the in vivo immunosuppression remains unclear.

INTRODUCTION

Parasitic infections of both man and domestic animals are highly prevalent throughout the world. More than half the world's human population is afflicted with parasitic helminths or protozoa. Parasites are important causes of disease in many countries; in addition, parasitism of domestic animals causes serious veterinary and economic problems. Unfortunately, the control of parasites by drug treatment or common health measures is frequently difficult, and prevention of infection by vaccination is currently not feasible.

Parasites usually induce an intense immune response in the host; nevertheless the parasites often survive. Furthermore, during parasitic infections, the ability of the host to respond to other, unrelated antigens is frequently impaired. Therefore, the host may be more susceptible to secondary infection with other pathogens or less able to respond favorably to immunization. Understanding how the parasite induces alterations of immune responses may be important in understanding how the parasite evades the host's immune responses and survives. This may also lead to understanding how effective immunity to the parasite can be enhanced, and consequently, may be important in the ultimate control of many parasitic infections.

Alterations in immune responses to unrelated antigens induced by a number of important protozoan parasites have been described, and possible mechanisms involved in these alterations have been investigated. Altered immune responsiveness has been reported in a few helminth infections, but

relatively little research has been done to determine the mechanisms underlying these alterations. The infection of mice with Trichinella spiralis has been well-characterized, and the immunity to the parasite has been studied extensively. The parasite has been reported to induce altered immune responses to several different antigens, but the mechanisms involved have not been identified. The purpose of this study was to describe the nature of altered immune responses in mice infected with T. spiralis, and to determine the mechanisms leading to this altered immune reactivity.

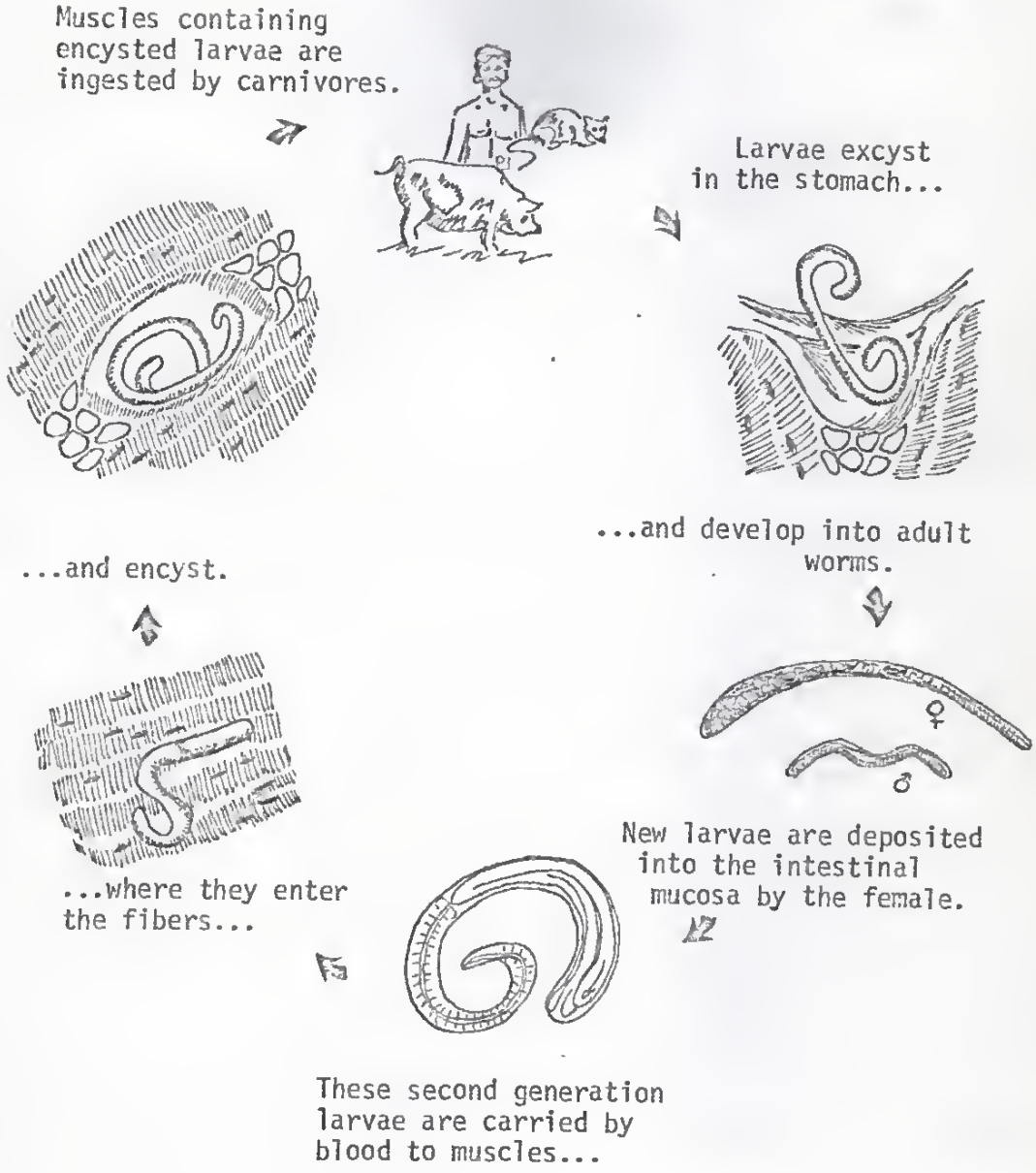


FIGURE 1. Life cycle of Trichinella spiralis.

BACKGROUND REVIEW

The Biology of *Trichinella spiralis*

Trichinella spiralis, which causes trichinosis, is a tissue-inhabiting nematode which commonly infects a variety of carnivorous and omnivorous mammals, including man. The general life cycle of *T. spiralis* is shown in Figure 1. Man usually becomes infected by eating poorly-cooked or raw pork containing encysted larvae, although in some parts of the world, wild animals such as bears are important sources of human infection.

The infection of mice with *T. spiralis* is a well-characterized experimental model (1, 2). A mouse ingests encysted larvae which are freed within a few hours by the action of digestive juices in the stomach. These larvae pass into the small intestine where the females mature within the mucosa. Second generation larvae, which are produced beginning four to five days postinfection, pass through the mucosa and travel from the intestinal lymphatics to the peripheral circulation. Circulating larvae are usually present by Day seven and begin penetrating muscle fibers where they undergo further development. Muscle larvae frequently can be found as early as seven days after infection, with the peak incidence of muscle penetration occurring during the third week of infection. By Day 30, muscle larvae are fully mature and infective. Intestinal adults are expelled from the gut apparently by a local inflammatory response beginning about 14 days after infection. Most adults have been eliminated by Day 30.

Immunity to Trichinella

The immune responses and the nature of acquired resistance to Trichinella have been studied extensively (reviewed in 3, 4). Crandall and Crandall described humoral and cellular responses of mice infected with T. spiralis (5). Larsh et al. showed that resistance to Trichinella, as measured by an accelerated expulsion of adults from the gut, could be transferred to normal mice by viable lymphocytes from infected or immunized mice, but not by serum (6, 7). Further evidence that the immune response to T. spiralis involves functional T-cells was provided by Walls et al. (8). They showed that in irradiated, thymectomized mice reconstituted with bone marrow, adult parasites persisted longer in the gut, and this persistence corresponded to a defect in the local inflammatory response of the gut. Furthermore, they found that more muscle larvae could be recovered from T-lymphocyte-deprived mice late in infection, and these mice had greater morbidity and mortality due to trichinosis. Perrudet-Badoux et al. reported that mice genetically selected for high and low antibody production had the same numbers of mature muscle larvae, indicating that antibody is not sufficient protection against infection (9).

Alterations of Immune Responsiveness Induced by Trichinella Infection

While infection with T. spiralis induced a significant immune response to the helminth, it also alters the ability of the host to respond immunologically to other, unrelated antigens. Both suppressed and enhanced immune responses to non-parasite antigens have been reported in infected mice.

Parasite-induced Immunosuppression

Effect on viral infections and immunity. One of earliest reports suggesting immune suppression to unrelated antigens following infection with Trichinella was by Kilham and Olivier who observed that rats infected with Trichinella 10 days prior to infection with encephalomyocarditis virus had higher crippling and death rates than rats given virus only (10). Similarly, Cypess and his co-workers demonstrated that mice infected with T. spiralis for seven days before infection with Japanese B encephalitis virus had higher death rates than mice receiving virus only. However, if they gave mice virus 28 days after Trichinella, the death rates of parasitized and control mice were the same. Also, they found that mice receiving virus up to 28 days after Trichinella had reduced primary and secondary complement-fixing antibody titers to the virus (11, 12). They suggested that decreased viral resistance could be due not only to alterations of the immune system, but also to physiologic alterations rendering the blood-brain barrier more permeable to virus (13).

Effect on allograft rejection. Svet-Moldavsky et al. showed that mice infected for 20 to 40 days with T. spiralis had significantly delayed rejection of skin allografts (14-16). These results were confirmed in a similar system by Faubert and Tanner, who also demonstrated that normal mice inoculated extensively with sera from infected mice had delayed allograft rejection (17).

Effect on immune responses to sheep erythrocytes. Most studies of suppressed immune responses induced by T. spiralis have employed sheep erythrocytes as the non-parasite antigen. In most experiments mice infected with T. spiralis were injected with sheep erythrocytes, and their antibody responses to sheep cells were determined either by serum hemagglutinin titers or by numbers of splenic antibody-forming cells.

In general, mice immunized with sheep erythrocytes during the first week of infection (Day 0 to 7) had antibody responses that were equal to those of unparasitized mice (18, 19). When immunized during the third or fourth week of infection (Day 15 to 30), mice had suppressed responses to sheep cells (12, 19, 20). If mice were immunized during the second week of infection (Day 8 to 14) they had either normal (12) or reduced (12, 19) anti-sheep erythrocyte responses, differences which may be explained by differences in the immunizing doses of sheep cells used, routes of immunization, or mouse strains employed. For example, Lubiniecki and Cypess found that when mice infected for 14 days were injected with sheep cells intravenously, their immune responses were the same as those of uninfected controls. When mice were immunized intraperitoneally, however, infected mice had significantly fewer splenic antibody-forming cells (12). This suppression was transient, since mice infected for more than 30 days at the time of immunization had normal response to sheep erythrocytes (12, 19).

Parasite-induced Immune Enhancement

Effect on resistance to Listeria infection. Cypess and co-workers showed that mice infected with Listeria monocytogenes intravenously 7 or 21 days after infection with Trichinella had higher LD-50s and longer survival times than control mice similarly infected with bacteria only (21, 22).

Effect on delayed hypersensitivity to BCG. Molinari et al. showed that mice infected with Trichinella for at least 14 days before immunization with BCG had increased responses to old tuberculin as assessed by 24 hour footpad swelling (23, 24). Interestingly, when they used heat-killed bacteria, they observed enhancement of footpad swelling following intraperitoneal or intravenous sensitization, but not after subcutaneous injection. They observed no such route dependency of enhancement if live bacteria were used.

When these same investigators infected mice with Trichinella 14 days after BCG immunization, they also found enhanced footpad swelling if responses were measured more than 20 days after Trichinella infection. However, if they measured the response to BCG 14 days after infection with Trichinella, they found suppressed responses, and this suppression could be transferred to uninfected mice with lymphoid cells (25).

Effect on tumor resistance. Weatherly reported that mice infected with Trichinella had lower incidences of spontaneous mammary tumors than comparable uninfected mice (26). Lubiniecki and Cypess studied the effect of Trichinella infection on experimental tumor growth in mice (27). They found that tumors grew more slowly in mice which had been infected with Trichinella 28 days before tumor inoculation, and infected mice had higher survival rates than unparasitized controls. The differences were small but statistically significant. Mice infected for 56 days were no different from unparasitized controls in their abilities to resist tumor growth.

Proposed Mechanisms of Altered Immune Responsiveness

A number of possible mechanisms have been proposed to explain alterations of immune responsiveness observed in mice infected with Trichinella, but few workers have tested their hypotheses. Some of the possible mechanisms include: (a) antigenic competition (11, 12, 19, 20); (b) functional or numerical alterations in cell populations (11, 21, 25, 28); (c) histological changes in lymphoid organs (29, 30); (d) the presence of antibody (20); (e) production of corticosteroids (11, 13); (f) altered ability to handle and process antigen (18); and (g) the presence of soluble suppressive substances derived from the parasites (11, 15-17, 19, 20, 28). These mechanisms are not mutually exclusive, and alterations in immune responsiveness are probably due to a combination of factors.

Antigen Competition

Simultaneous or sequential immunization with two different antigens may result in a suppressed immune response to one of the antigens. This phenomenon, first described by Michaelis (31), has been called antigenic competition or antigen-induced suppression (AIS) (reviewed in 32, 33). Suppression is usually observed if the two antigens are given at different times (34), or if given simultaneously, both are given in complete Freund's adjuvant (35); if both antigens are immunogenic (36, 37); and if they are given in the proper doses (32). Although the mechanisms of antigen-induced suppression are still not clear, sequential AIS is dependent on T-lymphocytes (38) and can be mediated by soluble factors (39-41) which may act via macrophages (40). Infection with T. spiralis induces T-dependent immune responses which may lead to AIS upon subsequent challenge with an antigen such as sheep erythrocytes.

Altered Cell Populations

The interactions of several cell types are necessary for the generation of most immune responses (42), but alterations in proportions can lead to suppression. For example, macrophages are required for most immune responses (43), but an excess of macrophages can lead to a decrease in immune responsiveness (44, 45). Kirchner et al. implicated macrophages as the cause of suppressed phytohemagglutinin (PHA) responses in splenocytes from mice with tumors induced by Moloney sarcoma virus (46). In contrast to their ability to suppress, macrophages were shown to enhance resistance to L. monocytogenes and Salmonella typhimurium in mice immunosuppressed during a graft-versus-host reaction, an immune response which was shown to induce hyperactive macrophages (47). Cypess et al. showed that 14 and 28 days postinfection (but not seven days), mice infected with Trichinella have increased rates of carbon clearance from the blood indicating the presence of activated, or at least phagocytic, macrophages (21). The increased resistance to Listeria and tumors and the enhanced delayed hypersensitivity to BCG could all be due to the presence of activated macrophages induced by Trichinella infection.

Suppressor cells -- mainly T-cells (reviewed in 48) but occasionally B-cells (49, 50) -- have been implicated in a number of systems in which suppression has been observed. Lubiniecki and Cypess measured the PHA responses of splenocytes from mice infected with T. spiralis for 7 12, or 21 days and found essentially normal responses. They concluded that there was no non-specific "defect" in T-cells from spleens of infected mice (12). However, the possible role of suppressor cells in Trichinella-induced suppression has not been carefully evaluated.

In attempting to delineate a cellula basis for immunosuppression, Faubert and Tanner thymectomized and irradiated normal mice and reconstituted them with (only) 1.3×10^5 bone marrow cells from normal mice or those infected with Trichinella for 30 days. Three weeks later, they injected these mice with sheep erythrocytes and measured their hemagglutinin titers seven days later. Mice reconstituted with bone marrow from infected mice had slightly lower hemagglutinin titers (mean 8, range 1 to 16) than those receiving normal bone marrow (mean 32, range 8 to 64). Fewer mice reconstituted with bone marrow from infected mice survived (3 survivors/10 mice in one experiment; 0/10 in another) as compared to those reconstituted with normal bone marrow (8 survivors/10 mice). Faubert and Tanner concluded that a "defect" was present in bone marrow cells from infected mice (28).

Another leukocyte prominent in mice infected with parasites such as Trichinella is the eosinophil (51). These cells have been shown to release prostaglandins (52, 53) which in turn have been shown to modulate immune responses (54, 55). The role of eosinophils in altering immune responses has not been established, although they have been shown to be important in immunity to schistosomes (56).

Altered Histology of Lymphoid Organs

Altered cell populations may be reflected in altered histology of the lymphoid organs themselves. Such histological changes could lead to alterations in cell traffic through the organ and/or prevent cell interactions necessary for an immune response. Faubert and Tanner showed that lymph nodes of infected mice increased in size during infection. Since the increase did not occur in thymectomized animals, they concluded that it was T-dependent (29). Molinari et al. described alterations in

the histology of the thymus in infected mice (31). Neither group has attempted to correlate these changes with alterations in function, so the importance of these changes in parasite-induced altered immune responses is unknown.

Suppression Induced by Antibody

Antibody-induced suppression, which probably acts by binding antigen and preventing a de novo immune response, has usually been shown to be specific for the antigen inducing its formation (57, 58). There is at least one report of antibody to one antigen suppressing the immune response to a different antigen, perhaps to undetected cross-reactivity between the two antigens (59). Similarly, immunosuppression due to antigen-antibody complexes appears to be specific (57). Some investigators have suggested that lymphocytes may be inhibited by cross-linked lattices through their Fc of C'-3 receptors (60, 61). However, Katz and Unanue showed that there is essentially no effect on antibody formation when cell surfaces are covered with antigen-antibody complexes (62).

The role of other immune mediators, the lymphokines, in both modulations of the immune response to T. spiralis and alterations of immune responses to other antigens has not been considered. Numerous products secreted by lymphocytes during an immune response have been described, usually on the basis of in vitro assays (63). In very few cases are the effects in vivo known. For example, interferon, one of the lymphokines, has been shown to reduce antibody formation (64, 65), delay skin graft rejection (66), and decrease delayed-type hypersensitivity (67). A recent report showed that mice treated with anti-interferon had increased susceptibility to encephalomyocarditis virus (68). If Trichinella infection inhibits interferon production, the host might be more

susceptible to viral infections. Conversely, if Trichinella induces interferon formation, this may affect skin graft rejection and formation of antibodies to other antigens.

Suppression by Corticosteroids

Corticosteroids have long been known to interfere with immunity. They have been shown to affect mainly B-cells (69, 70) and thymus cells (71), with less effect on long-lived, recirculating T-cells (69, 70). Stress has been shown to alter immune responsiveness through the effect of corticosteroids (72). Cypess et al. investigated the possibility that increased corticosteroid levels in Trichinella-infected mice were important in parasite-induced immunosuppression (11, 13). They demonstrated that infection with either Trichinella, which caused increased susceptibility to Japanese B encephalitis virus, and Nematospiroides dubius, which did not, resulted in similar elevations of serum corticosteroid levels. They concluded that elevated corticosteroid levels do not completely explain the increased susceptibility to the virus. The higher levels of cortisone might act to augment other, perhaps physiologic, alterations.

Distribution and Handling of Antigen

Cypess and co-workers showed that mice infected with Trichinella seven days prior to intravenous injection with [^{51}Cr]-sheep erythrocytes had reduced uptake of label by the spleen and increased uptake by the liver when compared to unparasitized mice similarly injected with labelled sheep cells. This may reflect increased activity by phagocytes in the liver (18). Greenwood et al. demonstrated that aggregated human gamma globulin failed to reach the germinal centers of mice immunosuppressed due to malarial infection (73). The data of Lubiniecki and

Cypess (12) and Molinari and Cypess (24) on the route dependency of altered immune responses to sheep erythrocytes and BCG support this hypothesis that parasitized animals are unable to process antigen as well as uninfected animals.

Production of Parasite-derived Suppressive Factors

The active production of soluble immunosuppressive substances during certain stages of Trichinella development has been proposed by Svet-Moldavsky et al.as an explanation of delayed allograft rejection in infected mice (14-16). Faubert and Tanner supported this hypothesis by demonstrating that injection of saline extracts of T. spiralis into normal mice suppressed the formation of sheep erythrocyte rosette-forming cells (20). Similarly, Barriga showed that injection of T. spiralis extract into normal mice inhibited the formation of anti-sheep cell hemagglutinating antibody (74). Faubert also reported that newborn larvae, but not muscle larvae or adults, could inhibit the production of anti-sheep erythrocyte plaque-forming cells following in vitro immunization of splenocytes from normal mice (19). That such parasite-derived suppressive factors might occur in the serum was shown by Faubert and Tanner, who demonstrated inbibition of skin graft rejection in normal mice inoculated with sera from mice infected for 30 days with Trichinella (17). They also showed that sera from mice infected with Trichinella had leukoagglutinin and leukotoxic activity. They concluded that, due to the kinetics of appearance of this substance, it was not antibody (17).

Alterations of Immune Responsiveness Induced by Other Parasites

Alterations of immune responses to unrelated antigens have been observed following infection with a variety of other parasites including protozoa (75, 76), trematodes (77), cestodes (78), and other nematodes (79-81). The effect of protozoan infection on immune responsiveness has been studied most extensively.

Although many mechanisms have been proposed to explain these alterations and there is good experimental evidence for some of the hypotheses, the "immunological defect" underlying most of these immune alterations remains to be established. Altered macrophage function has been demonstrated in malarial (75), trypanosomal (75), and Nippostrongylus infections (82), and may be important in inducing alterations of immune responsiveness. Suppressed mitogen responses have been demonstrated following infection with Toxoplasma (83) and schistosomes (84). In the case of schistosome infections, the authors considered suppressor T-cells to be important (84). During malarial infection, alterations in splenic histology have been demonstrated; they are probably important in the immunosuppression associated with that disease (75). In most cases of parasite-induced immunosuppression, antigen-induced suppression (antigenic competition) has been considered a probable cause of that suppression, but this has not been demonstrated experimentally (75, 77, 78).

Statement of the Problem

The interactions that can lead to alterations of an animal's ability to respond immunologically to an antigen are complex. While there are numerous reports of altered immune responsiveness in animals infected with Trichinella, few of these phenomena have been examined to determine their underlying causes. The purpose of this study was to investigate

the immune responses to sheep erythrocytes in mice infected with Trichinella, using primarily a system of in vitro immunization to examine the mechanisms of parasite-induced alterations of immune responsiveness to a second antigen.

MATERIALS AND METHODS

Mice

C57Bl/6J female mice (Jackson Laboratories, Bar Harbor, Me.) 6 to 12 weeks old were used in most of the experiments reported here. CD-1 female mice (Charles River, Wilmington, Mass.) five to eight weeks old were used to assay delayed hypersensitivity. Mice were housed in the Animal Resources Division of the University of Florida Medical Center, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Antigens

Sheep Erythrocytes (SRBC)

Most experiments used H-type sheep red blood cells (SRBC) (85), obtained from a single sheep (Colorado Serum Co., Denver, Colo.). The cells were washed three times in Dulbecco's phosphate-buffered saline (Dulbecco's PBS) (86) and stored for up to three months in the medium described by Click et al. (87). In specified experiments, pooled SRBC (BBL, Cockeysville, Md.) washed three times in PBS were used for in vivo immunization and delayed hypersensitivity.

DNP--Ficoll

Dinitrophenol (DNP) was coupled to Ficoll as described in detail by Sharon et al. (88). Ficoll (ave MW 4 x 10^4 daltons; Pharmacia, Upsala, Sweden) was dissolved in water to which NaOH and $KHCO_3$ were added. Cyanuric chloride (trichloro-s-triazine; Eastman Organic Chemicals, Rochester, N. Y.) suspended in dimethyl formamide was added

to the Ficoll solution. ε-2,4 dinitrophenol-L-lysine (Sigma, St. Louis, Mo.) was dissolved in water, adjusted to pH 11 with NaOH, added to the Ficoll-cyanuric chloride mixture, and stirred overnight. After extensive dialysis against water and saline, this DNP-Ficoll was passed through a 0.22 μ Millipore filter and stored at 4°C. The carbohydrate content was measured by the phenol-sulfuric acid method of Dubois et al. (89), and the concentration of ε-DNP-lysine groups was measured spectrophotometrically (λ = 365 nm, E = 16,400). The preparation used in these experiments contained 52 moles of DNP-lysine per 4 x 10^4 MW units of carbohydrate.

Trichinella Extract

Trichinella larvae which had been stored frozen in saline were suspended in Hanks' Balanced Salt Solution (HBSS) and passed repeatedly through a French pressure cell until no whole larvae were visible under a dissecting microscope. This material was centrifuged 30 minutes at 300 g and the supernatant was concentrated by negative dialysis. This concentrated supernatant was analyzed for protein concentration by the Lowry method (90) and stored frozen.

Trichinella Secretory Products

Fresh larvae, which had been washed once in saline, were suspended in RPMI-1640 medium and incubated at 37°C for four to eight hours. The larvae were allowed to settle in conical centrifuge tubes and the supernatant fluid was removed by aspiration. The supernatant was concentrated, analyzed for protein content, and stored frozen.

Immunization Procedures

Trichinella Infection

Mice were routinely infected with 200 T. spiralis larvae via stomach intubation by the method of Larsh and Kent (91) using Trichinella obtained in 1960 from Dr. John E. Larsh (Department of Parasitology, University of North Carolina, Chapel Hill, N. C.) and maintained continuously in rats and mice in this laboratory.

In Vivo Immunization

Sheep erythrocytes. Spleens were immunized by the intraperitoneal injection of two to 4 x 10^8 sheep erythrocytes in HBSS or saline (92, 93). Lymph nodes were immunized by the subcutaneous injection of 10^8 SRBC into each hind or front footpad (94). Five days after immunization, which was shown to be the optimal time for measuring AFC in C57Bl/6 mice (95), spleens or draining lymph nodes were removed and assayed for the number of antibody-forming cells (AFC).

DNP-Ficoll. Varying amounts of DNP-Ficoll were injected intraperitoneally and seven days later spleens were removed and assayed for AFC.

In each experiment, five to eight mice were immunized per group and two to three unimmunized, parasitized mice were used as background controls. Spleens or lymph nodes were pressed gently through wire screens into cold HBSS. Single cell suspensions were prepared by gently expelling cells from a syringe through successively smaller needles from 20 to 25 gauge. Cells were then washed once and suspended to the desired concentration in HBSS and assayed for AFC.

^{125}I was enzymatically coupled to SRBC using lactoperoxidase (96). Forty microliters of a 10% suspension of labelled SRBC were injected into each hind footpad of normal or 20-day infected mice. Mice were maintained on tap water containing KI and NaCl. At various times after injection of antigen mice were killed, and selected organs were removed, weighed, and assayed for radioactivity in a well-type gamma counter. Blood volume was estimated to be 77.8 ml/kg/mouse (97).

In Vitro Immunization

Lymphocytes were immunized in vitro by the method of Click et al. (87). Spleens or lymph nodes from five to 25 mice were removed aseptically and were pressed gently through wire screens into cold Dulbecco's PBS plus antibiotics. The cells were transferred by pipette to a sterile centrifuge tube and were allowed to settle on ice for 5 minutes. The single cells suspended in the supernatant were transferred to a second sterile tube and were washed three times in cold Dulbecco's PBS. After lysing red blood cells with warm NH_4Cl, the cell suspension was washed again in Dulbecco's PBS. (In all washing steps, lymph node cells were washed in Dulbecco's PBS containing fetal calf serum.) Viable cells were enumerated by trypan blue exclusion and lymphocytes were suspended to the desired concentration in culture medium. Eight to ten million viable cells in 0.1 ml culture medium were distributed into sterile 35 x 10 mm plastic culture dishes (Falcon #3001; Scientific Products, Ocala, Fl.) containing 2 ml culture medium and 0.1 ml antigen. Cultures were kept in gas-tight boxes and were gassed daily with a mixture of 5% CO_2, 12% O_2, and 83% N_2.

After four days incubation with DNP-Ficoll or five days with SRBC, cells were harvested by scraping culture dishes gently with a rubber

policeman. The cells were washed once in Dulbecco's PBS, resuspended in HBSS plus NaOH (pH 7.0 to 7.2) and assayed for antibody-forming cells. Since cell recovery in experimental and control groups within a single experiment was always similar, data are presented as AFC per culture. Similar conclusions could be reached using data calculated as AFC per 10^6 recovered, viable cells.

Supernatant fluids. Splenocytes were cultured for four days in the absence of any exogenous antigen. Cultures were pooled into 50 ml centrifuge tubes and centrifuged; the supernatants were stored frozen. Before use, supernatant fluids were concentrated 10 times by negative pressure dialysis and passed through 0.22 μ Millipore filters.

Measurements of Immune Responses

Antibody-forming Cells (AFC)

Direct (IgM) anti-sheep erythrocyte AFC were measured by a slide modification (85) of the Jerne plaque assay (98) using guinea pig complement (GIBCO, Grand Island, N. Y.). Anti-DNP antibody-forming cells were measured similarly using SRBC to which DNP had been coupled as the assay cells.

DNP-coupled Sheep Erythrocytes

DNP was coupled to Fab' fragments of rabbit anti-sheep erythrocyte antiserum by Dr. Catherine Crandall (Department of Pathology, University of Florida). IgG, isolated by DEAE-cellulose chromatography from the sera of rabbits immunized repeatedly with SRBC, was digested with pepsin and the fragments were reduced with 2-mercaptoethanol and alkylated with iodoacetomide (99, 100). Fab' fragments, separated on a Sephadex G-200 column, were incubated with DNP-sulphonic acid, and the resulting DNP-Fab

fragments were purified on a G-200 column. These DNP-fabs were incubated with a suspension of SRBC for one hour at 37°C (101).

Serum Hemagglutinin (HA) Titers

Mice were exsanguinated and their sera stored frozen. Sera were assayed for 2-mercaptoethanol-sensitive (IgM) and -resistant (IgG) antibody titers using microtiter methods (102, 103). The HA titer was the reciprocal of the highest dilution showing positive hemagglutination.

Delayed-type Hypersensitivity Assays

Sheep erythrocytes. Delayed hypersensitivity to SRBC was determined in CD-1 mice using the method of LaGrange et al. (104). Six to seven infected or normal mice were sensitized via the spleen by intravenous injection of varying doses of pooled SRBC; other mice were sensitized via the lymph nodes by subcutaneous injection of 10^7 SRBC into the right hind footpad. Four days after intravenous or five days after subcutaneous sensitization, mice were challenged in the left hind footpad with 10^8 SRBC. Twenty-four hours later footpad thicknesses were measured using a Starett microcaliper (Starett Instrument Co., Athol, Mass.). Four to five mice were challenged but NOT sensitized and served as background controls.

Oxazalone. Contact hypersensitivity to oxazalone was determined by the method described by Fiske and Klein (105). Mice were sensitized to oxazalone (2-phenyl-4-ethoxymethylene oxazalone; BDH, Ltd., London, England) by applying 0.1 ml of a 3% solution of oxazalone in absolute ethanol to the shaved abdomens. Eight days later, contact hypersensitivity to oxazalone was assessed by measuring the 24 hour increase in ear thickness of mice challenged on the ear with a cotton swab soaked in 3% oxazalone in olive oil. Control mice were sensitized with ethanol only.

Determinations of Cell Populations

Differential Cell Counts

Lymphocytes suspended in RPMI-1640 medium plus protein (bovine serum albumin (BSA) or fetal calf serum at a final concentration of at least 1%) were incubated with colloidal carbon for 30 minutes at 37°C. After repeated washings, cells were pelleted on microscope slides with a cytocentrifuge (Shandon Scientific Co., Ltd., London, England) and stained with May-Gruenwald Giemsa stain. Differential cell counts were made of at least 200 cells per slide.

Cytotoxic Assays

Anti-thy 1.2 antiserum (donated by Dr. Bryan Gebhardt and Dr. James Forbes, Department of Pathology, University of Florida) was produced in CBA mice by repeated injections of thymocytes from young (4 week old) AKR mice (106). Rabbit anti-mouse immunoglobulin (Ig) (donated by Dr. Catherine Crandall) was prepared by injection of rabbits with an antigen-antibody precipitate of sera from mice infected with *Ascaris suum* (107), and was shown by immunoelectrophoresis to recognize all mouse Ig classes.

One million lymphocytes were added to wells of microtiter trays containing serially diluted antiserum or control normal serum and guinea pig complement. Following incubation at 37°C for 45 minutes, cells were assayed for viability by trypan blue exclusion.

Complement-dependent Rosettes

Complement-receptor-bearing lymphocytes were enumerated by the bacteria-antibody-complement (BAC) rosette assay described by Gormus *et al.* (108). An overnight culture of *Salmonella typhimurium* was incubated with a dilution of anti-*S. typhimurium* antibody. This complex, designated BA (bacterial-antibody), was incubated with fresh normal mouse serum as a

source of mouse complement to yield BAC. BA and BAC were washed repeatedly, suspended in RPMI-1640 containing bovine serum albumin and stored at -70°C. Lymphocytes suspended in RPMI-1640 plus BSA were incubated with BA or BAC at 0°C for 45 minutes. After extensive washing, cells were pelleted on microscope slides with a cytocentrigue, stained with May-Gruenwald Giemsa stain, and counted for the proportion of rosetting cells. Four hundred cells were counted per slide. Cells binding three or more bacteria were recorded as positive.

Mitogen Stimulation

Single cell suspensions of spleens or lymph nodes were prepared aseptically as described above ("In Vivo Immunization"). After being washed twice in HBSS, the cells were adjusted to the desired concentration in RPMI-1640 medium containing 5% human serum and antibiotics.

Pooled lymphocytes from two to six animals were cultured in the presence of the T-cell mitogens, Concanavalin A (Con A; Miles Laboratories, Kankakee, Ill.) and phytohemagglutinin (PHA; PHA-P, Difco Laboratories, Detroit, Mich.), or the B-cell mitogen, lipopolysaccharide (LPS; S. typhimurium LPS-W, Difco) using microculture methods (109). Five hundred thousand cells were cultured in quadruplicate wells of U-shaped microculture plates (Linbro Chemical Co., Inc., New Haven, Conn.) for 72 hours with 0.5 μCi per well of tritiated thymidine (methyl-^{3}H, sp. act. 1.9 Ci/mMole; Schwarz/Mann, Orangeburg, N.Y.) present during the final 24 hours of culture. Cells were harvested mechanically (Hiller Harvester, Otto Hiller Co., Madison, Wisc.) and the radioactivity in an acid insoluble product was measured in a liquid scintillation counter. Data are reported for the optimal doses of mitogen which were as follows: PHA, 0.25 μl per culture; Con A, 0.5 μg per culture; and

LPS, 5.0 μg per culture. LPS was boiled in phosphate buffer (pH 8.0) for one hour before use (110).

Splenectomy

Mice were anesthetized with ether and their left flanks shaved. A 1 to 2 cm incision was made on the flank above the spleen, the hilum was ligated with a silk suture, and the spleen was cut away and discarded. The peritoneum was closed with a single silk suture, and the skin with two metal wound clips. In sham operations, the spleen was exposed as above and then pushed gently back into the abdomen.

Statistical Analysis

Data were analysed by Student's *t*-test, using log transformations of AFC data. Means were considered different with a 95% confidence limit ($p \leq 0.05$).

RESULTS

Immune Responses to Non-parasite Antigens

In Vivo Immunization with Sheep Erythrocytes

The immune response to sheep erythrocytes (SRBC) following systemic immunization was assessed in mice which had been infected with Trichinella for different lengths of time. The number of splenic antibody-forming cells (AFC) and the serum hemagglutinin (HA) titers were determined five days after the intraperitoneal injection of SRBC. The data shown in Table 1 indicate that significant suppression of the direct AFC responses of infected mice was demonstrable 20 days after infection. Likewise, the serum HA titers before and after reduction with 2-mercaptoethanol were lower in infected mice 20 days after infection (Table 2).

To investigate the immune response to SRBC following local immunization, mice which had been infected 20 days earlier were immunized by injecting SRBC into the hind or front footpads. Five days later the draining lymph nodes were assayed for numbers of direct AFC. Infected mice developed fewer AFC than uninfected controls (Table 3), which correlates with the suppression observed following systemic immunization. Although the responses of axillary and brachial lymph nodes from infected mice were only 50% of normal, the differences were not statistically significant due to the very large standard error.

In Vitro Immunization with Sheep Erythrocytes

Since infected mice had reduced antibody responses to sheep erythrocytes following *in vivo* immunization, lymphocytes from infected

TABLE 1

ANTIBODY-FORMING CELLS IN SPLEENS FOLLOWING IMMUNIZATION IN VIVO WITH SHEEP ERYTHROCYTES

DAYS POST-INFECTION[a]	AFC/WHOLE SPLEEN		AFC IN INFECTED MICE AS PERCENT OF CONTROL
	CONTROL	INFECTED	
3	32,500 ± 5,600	26,600 ± 5,400	82
3	50,200 ± 11,200	36,300 ± 7,000	72
7	38,800 ± 800	38,100 ± 3,100	98
7	33,300 ± 12,100	29,800 ± 3,900	89
14	54,400 ± 12,000	64,200 ± 3,900	118
14	34,300 ± 1,900	33,900 ± 4,900	93
20[b]	80,470 ± 10,405	38,320 ± 6,210	48[c]
20	55,558 ± 8,549	29,420 ± 9,180	53[c]

a. Five normal or infected mice per group. AFC were assayed five days after intraperitoneal injection of 0.2 ml of a 10% SRBC suspension. Mean ± sem. Three parasitized mice not immunized with SRBC served as background controls; none of these mice had any splenic AFC.

b. Mice immunized with H-type SRBC. All others immunized with pooled SRBC.

c. Responses of infected mice lower than controls, $p < 0.05$.

TABLE 2

SERUM HEMAGGLUTININ TITERS FOLLOWING INTRAPERITONEAL IMMUNIZATION WITH SHEEP ERYTHROCYTES

DAYS POST-INFECTION[a]	LOG_2 HA TITER			
	BEFORE REDUCTION WITH 2-ME		AFTER REDUCTION WITH 2-ME	
	CONTROL	INFECTED	CONTROL	INFECTED
3	7.6 ± 0.4	6.2 ± 0.2	3.2 ± 0.9	2.4 ± 0.5
3	13.6 ± 0.7	11.8 ± 0.2	8.6 ± 0.2	7.4 ± 0.4
7	6.5 ± 0.3	7.0 ± 0.6	2.2 ± 0.7	2.5 ± 0.5
7	16.8 ± 1.6	12.8 ± 1.3	10.2 ± 0.5	9.8 ± 0.8
14	6.2 ± 0.2	6.2 ± 0.2	1.2 ± 0.2	1.3 ± 0.2
14	10.4 ± 1.1	8.8 ± 0.5	3.4 ± 0.7	3.2 ± 0.5
20	9.6 ± 0.4	7.2 ± 0.2[b]	5.2 ± 0.2	3.6 ± 0.2[b]
20	8.6 ± 0.7	6.5 ± 0.5[b]	5.8 ± 0.5	4.5 ± 0.3

a. Sera from mice described in Table 1. Mean ± sem.

b. HA titers of infected mice lower than control mice, $p < 0.05$.

TABLE 3

ANTIBODY-FORMING CELLS IN LYMPH NODES FOLLOWING IMMUNIZATION IN VIVO WITH SHEEP ERYTHROCYTES

LYMPH NODES	MICE		CELLS PER SET OF LYMPH NODES ($\times 10^{-6}$)	AFC PER 10^6 CELLS[c] (% OF CONTROL)[d]	AFC PER MOUSE (% OF CONTROL)[d]
	GROUP[b]	NUMBER			
Popliteal	Control	7	5.2 ± 0.3	1,526 ± 672	5,257 ± 1,118
	Infected	7	4.3 ± 0.4[e]	405 ± 82[e] (26%)	1,943 ± 445[e] (37%)
Popliteal	Control	6	6.1 ± 0.4	1,219 ± 312	7,907 ± 2,146
	Infected	5	3.3 ± 0.6[e]	352 ± 187[e] (29%)	1,440 ± 898[e] (18%)
Axillary & Brachial	Control	6	11.9 ± 0.8	1,876 ± 476	17,750 ± 5,086
	Infected	8	11.4 ± 0.7	1,019 ± 238 (54%)	9,138 ± 2,200 (51%)
Brachial	Control	5	13 ± 0.6	3,712 ± 978	25,838 ± 11,689
	Infected	6	12.1 ± 1.3	1,982 ± 387 (53%)	9,560 ± 2,340 (37%)

a. Mice were injected into each hind or front footpad with 10^8 SRBC and five days later draining lymph nodes were assayed for AFC. Means $\pm$ sem. Popliteal nodes were assayed following hind footpad injection; axillary and brachial were assayed following front footpad injection.

b. Infected mice had been infected 20 days previously.

c. Based on viable cell counts. Cells were $\sim$80% viable.

d. % of control = [(AFC, infected mice) $\div$ (AFC, control mice)] X 100.

e. Infected mice lower than controls, $p < 0.05$.

mice were assessed for their ability to respond to SRBC *in vitro*. Splenocytes from normal or infected mice were immunized with SRBC *in vitro*. Following five days in culture, the cells were assayed for numbers of direct AFC. These results, shown in Table 4, demonstrate that suppression was found only 20 days postinfection, which correlates with the results obtained following *in vivo* immunization. In another experiment, individual spleens from normal or 20-day infected CD-1 mice were immunized *in vitro* with SRBC. In this experiment, spleens from infected mice had significantly lower numbers of AFC than did splenocytes from normal mice.

To determine if suppression of the *in vitro* response to SRBC was dependent on the infecting larval dose, groups of five mice were infected with either 0, 50, 200, or 400 *T. spiralis* larvae. Twenty days later, splenocytes from these mice were immunized with SRBC *in vitro*. As shown in Fig. 2, the dose of *Trichinella* used for infection had no effect on the degree of suppression observed *in vitro*. In all other experiments, mice were infected with 200 *Trichinella* larvae.

Lymph node cells were immunized to SRBC *in vitro* and after five days, they were assayed for numbers of direct AFC. As shown in Table 5, lymph node cells from infected mice developed more AFC than did those from normal mice. This enhancement was observed in axillary and brachial lymph node cells from mice infected for 20 days as well as in cells from the mesenteric lymph nodes of mice infected for 7 or 14 days.

In Vivo Immunization with DNP-Ficoll

Immunity to the T-independent antigen, DNP_{52}-Ficoll, was determined in mice which had been infected with *Trichinella* 20 days prior to

TABLE 4

ANTIBODY-FORMING CELLS IN SPLENOCYTE CULTURES FOLLOWING IMMUNIZATION IN VITRO WITH SHEEP ERYTHROCYTES

DAYS POST-INFECTION[a]	AFC/CULTURE		AFC IN SPLENOCYTES FROM INFECTED MICE AS PERCENT OF CONTROL
	CONTROL	INFECTED	
7	2,535 ± 250	4,080 ± 170	161
7	2,385 ± 1,020	2,297 ± 360	96
14	2,535 ± 250	2,875 ± 250	113
21	5,480 ± 450	900 ± 80	16[b]
21	6,505 ± 720	765 ± 90	12[b]

a. Splenocytes from normal or infected mice were cultured for five days in the presence of SRBC. Mean ± sem of four replicate cultures.

b. Responses of cells from infected mice significantly lower than controls, $p < 0.05$.

FIGURE 2. Effect of varying the numbers of larvae used for infection on the generation of AFC *in vitro*. Mice were infected 20 days before *in vitro* immunization with SRBC. Mean ± sem of four replicate cultures. The responses of cells from infected mice were always lower than those from normal mice ($p < 0.05$).

FIGURE 2. Effect of varying the numbers of larvae used for infection on the generation of AFC *in vitro*. Mice were infected 20 days before *in vitro* immunization with SRBC. Mean ± sem of four replicate cultures. The responses of cells from infected mice were always lower than those from normal mice ($p < 0.05$).

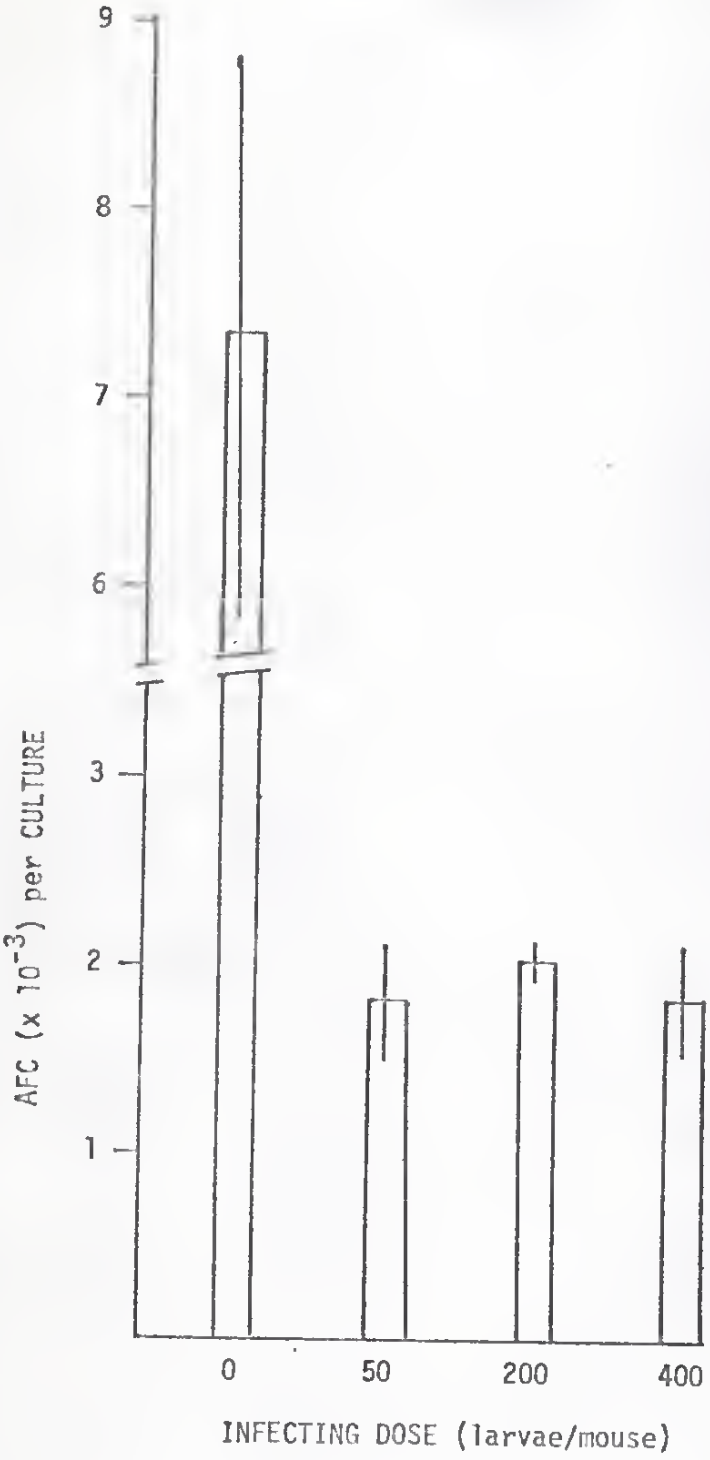

9
8
7
6
3
2
1
AFC (x 10^{-3}) per CULTURE
0
50
200
400
INFECTING DOSE (larvae/mouse)

TABLE 5

ANTIBODY-FORMING CELLS IN LYMPH NODE CELL CULTURES FOLLOWING IMMUNIZATION *IN VITRO* WITH SHEEP ERYTHROCYTES

DAYS POST-INFECTION	LYMPH NODES USED	AFC/CULTURE[a]		AFC IN CELLS FROM INFECTED MICE AS PERCENT OF CONTROL
		CONTROL	INFECTED	
7	Mesenteric	1,875 ± 85	4,513 ± 482	240[b]
14	Mesenteric	3,895 ± 465	13,775 ± 2,194	354[b]
21	Axillary and Brachial	45 ± 26	1,030 ± 257	2,289[b]
21	Axillary and Brachial	250 ± 70	1,060 ± 206	424[b]
21	Axillary and Brachial	2,425 ± 200	4,870 ± 1,030	201[b]

a. Lymph node cells were cultured with SRBC for five days. Mean ± of four replicate cultures.

b. Responses of cells from infected mice significantly higher than controls.

intraperitoneal injection with 80 or 800 µg of DNP-Ficoll. Seven days after immunization with DNP-Ficoll, the spleens of these mice were assayed for anti-DNP antibody-forming cells. As shown in Table 6, infected mice developed more AFC than normal mice, but the differences were not always statistically significant. Passive HA titers were usually similar in infected and normal mice (Table 7).

Subcutaneous immunization to stimulate the draining lymph node was attempted by injection of 80 µg of DNP-Ficoll into each hind footpad. When the popliteal lymph nodes were assayed for AFC seven days later, no AFC were observed. The failure of lymph nodes to respond to this immunogen has been reported by other investigators (111).

In Vitro Immunization with DNP-Ficoll

Splenocytes from normal or 20-day infected mice were cultured for four days with DNP-Ficoll and were assayed for numbers of anti-DNP antibody-forming cells. Splenocytes from infected mice had significantly higher numbers of direct AFC than splenocytes from normal mice (Fig. 3).

Delayed-type Hypersensitivity

The antibody responses to sheep erythrocytes and DNP-Ficoll were altered in mice infected with T. spiralis, suggesting an effect on the humoral immune system in infected mice. To determine if cellular immunity was also affected by Trichinella infection, mice were assessed for delayed hypersensitivity to SRBC and contact hypersensitivity to oxazalone.

Spleen-mediated delayed hypersensitivity to SRBC was measured in normal or 20-day infected CD-1 mice which had been sensitized to SRBC by intravenous injection of varying doses of pooled SRBC. Four days after sensitization, mice were challenged in the left hind footpad, and

TABLE 6

ANTI-DNP ANTIBODY-FORMING CELLS IN SPLEENS FOLLOWING IMMUNIZATION *IN VIVO* WITH DNP-FICOLL

EXPERIMENT	IMMUNIZING DOSE	AFC/WHOLE SPLEEN		AFC IN INFECTED MICE AS PERCENT OF CONTROL
		CONTROL	INFECTED	
A	0[b]	0	750 ± 750	
	80 µg	30,500 ± 4,680	45,300 ± 13,160	148
	800 µg	13,900 ± 1,600	25,580 ± 3,015	188[c]
B	80 µg	4,720 ± 1,555	10,380 ± 2,035	220[c]
	800 µg	2,140 ± 590	3,340 ± 795	156

a. Six 20-day infected or control (uninfected) mice were injected intraperitoneally with DNP-Ficoll in saline and spleens were assayed for AFC seven days later. Mean ± sem.

b. Four control and four infected mice.

c. Infected mice had significantly more AFC than controls, $p < 0.05$.

TABLE 7

SERUM HEMAGGLUTININ TITERS TO DNP FOLLOWING IMMUNIZATION IN VIVO WITH DNP-FICOLL

EXPERIMENT[a]	IMMUNIZING DOSE	TITER (LOG_2)	
		CONTROL	INFECTED
A	0	3.7 ± 0.3	5.3 ± 0.8
	80 µg	10.5 ± 0.3	10.7 ± 0.7
	800 µg	9.0 ± 0.2	10.8 ± 0.4[b]
B	0	3.0 ± 2	1.0 ± 0
	80 µg	12.0 ± 0.6	11.5 ± 0.7
	800 µg	9.2 ± 0.6	11.2 ± 1

a. Mice as described in Table 6.

b. HA titers of infected mice higher than controls, $p < 0.05$.

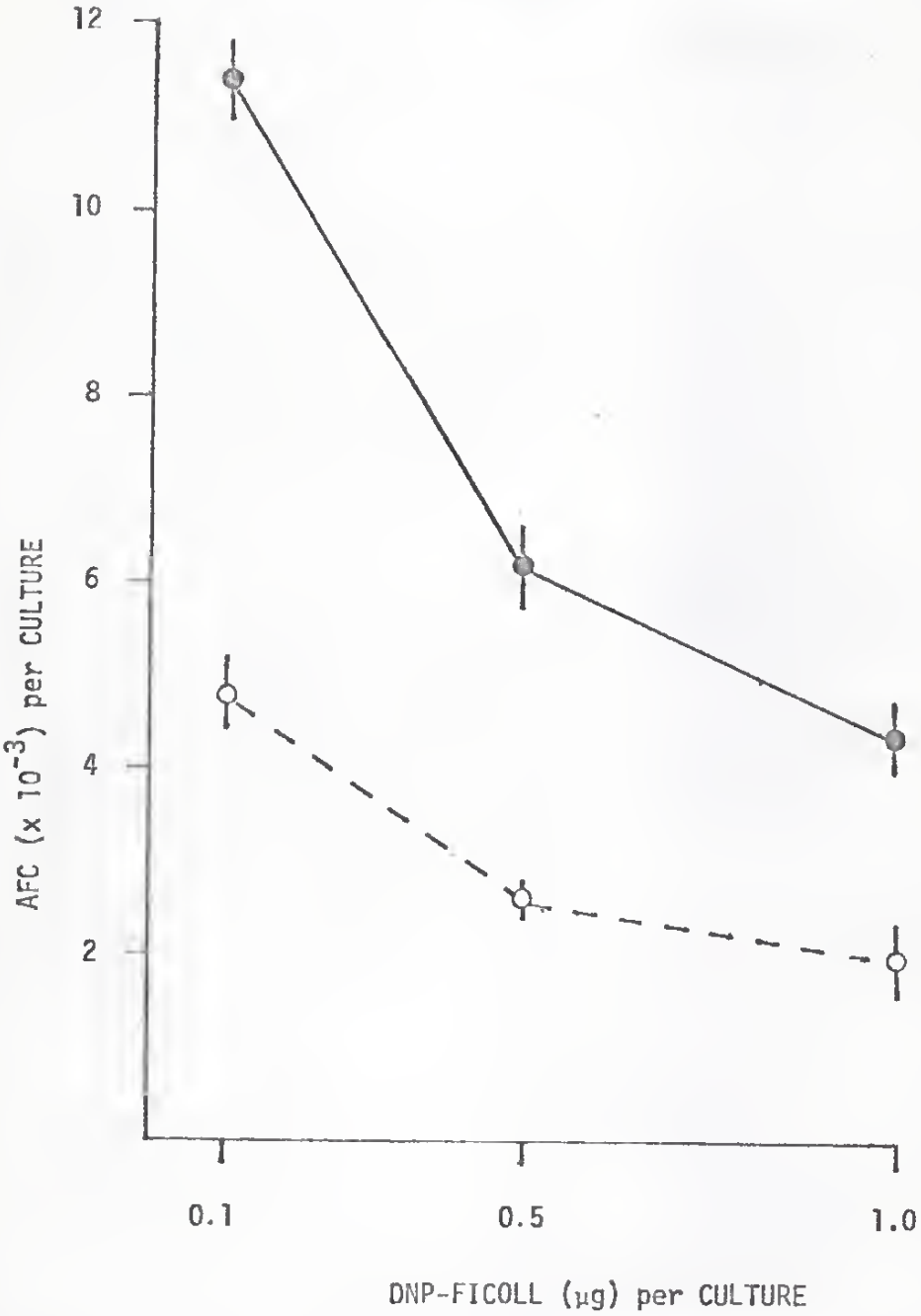


FIGURE 3. AFC in splenocyte culture following in vitro immunization with DNP-Ficoll. Cells were cultured for four days. Mean ± sem of four replicate cultures. ○ Cells from normal mice. ● Cells from mice infected for 20 days. In all cases, the responses of cells from infected mice were significantly higher than normal ($p < 0.05$).

the thickness of their footpads were measured 24 hr later. The results, shown in Fig. 4, indicate that infected and normal mice had similar delayed hypersensitivity responses to SRBC. However, the differences in footpad swellings between unsensitized, parasitized mice and optimally sensitized (10^5 SRBC), parasitized mice were only marginally significant ($0.1 > p > 0.05$). Similar results were obtained in a second experiment except that the responses to normal and infected mice not sensitized to SRBC were the same, and the footpad swellings of parasitized mice which had been sensitized with 10^5 SRBC were significantly higher than those of unsensitized, parasitized mice.

Lymph node-mediated delayed hypersensitivity was determined in CD-1 mice sensitized to SRBC by injecting SRBC subcutaneously into the left hind footpads 20 days after infection with Trichinella. Five days after sensitization, mice were challenged in the right hind footpad, and footpad swellings were measured 24 hours later. The data show that normal and infected mice had similar responses to SRBC (Fig. 5). A second experiment gave similar results.

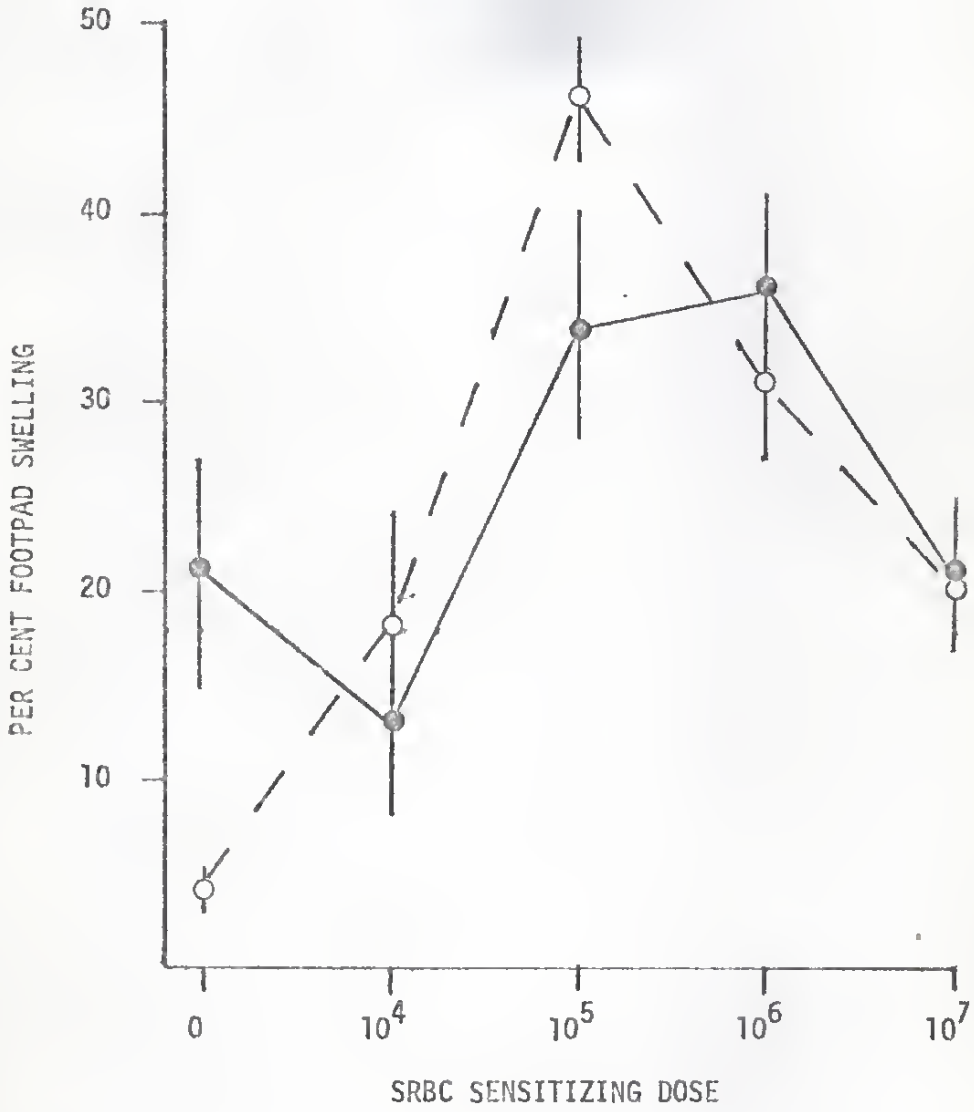
The ability of mice to respond to the contact sensitizing agent oxazalone was assessed in normal or infected CD-1 mice. Eight days after primary exposure to oxazalone, mice were tested for contact hypersensitivity. The results are shown in Table 8. Normal mice or those infected for 20 days at the time of sensitization had similar responses upon subsequent challenge with oxazolone. However, infected mice challenged but NOT sensitized, had significantly greater ear swelling than did unparasitized, unsensitized controls.

Mechanisms of Splenic Immunosuppression

Mice infected with Trichinella for 20 days had reduced antibody

FIGURE 4. Delayed hypersensitivity mediated by the spleen. Mice were infected 20 days prior to intravenous sensitization with SRBC. They were challenged four days later and footpad thicknesses were measured after 24 hours. Mean $\pm$ sem. ○ Normal mice. ● Infected mice. Data are percent footpad swelling:

$$\frac{\text{thickness left footpad} - \text{thickness right footpad}}{\text{thickness right footpad}} \times 100$$


50
40
30
20
10
PER CENT FOOTPAD SWELLING
0
10^4
10^5
10^6
10^7
SRBC SENSITIZING DOSE

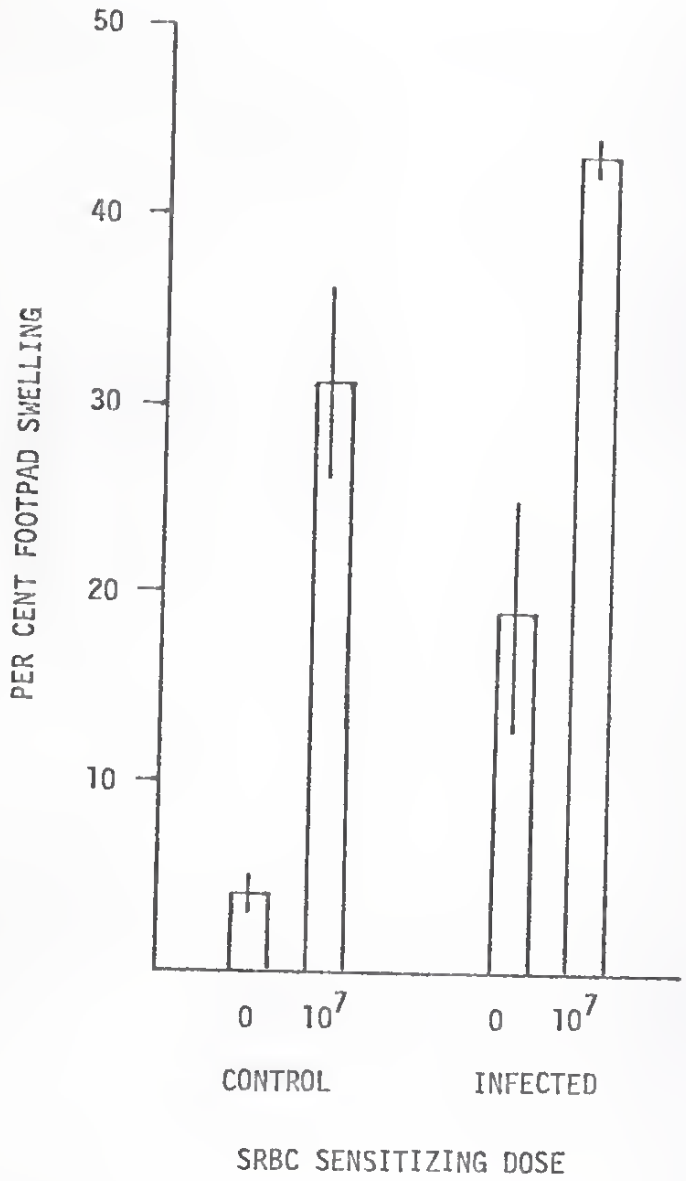


FIGURE 5. Delayed hypersensitivity mediated by lymph nodes. Mice were infected 20 days prior to subcutaneous sensitization. They were challenged five days later and footpad thickness was measured after 24 hours. Mean ± sem. Data are percent footpad swelling:

$$\frac{\text{thickness after challenge} - \text{thickness before challenge}}{\text{thickness before challenge}} \times 100$$

TABLE 8

SENSITIVITY TO OXAZALONE

MICE[a]	PERCENT SWELLING[b]	
	UNSENSITIZED (N)	SENSITIZED (N)
Control	31 ± 14 (4)	109 ± 17 (8)
Infected	77 ± 7 (2)	109 ± 13 (8)

a. Unparasitized control mice or those infected with Trichinella for 20 days were sensitized to oxazalone and challenged eight days later.

b. Mean ± sem of the indicated number (N) of mice.

Percent swelling =

$$\frac{\text{ear thickness after challenge-ear thickness before challenge}}{\text{ear thickness before challenge}} \times 100$$

responses to SRBC following *in vivo* and *in vitro* immunization. The experiments described below were designed to determine the underlying mechanisms of this suppression. Unless specifically stated otherwise, all work reported below was carried out with splenocytes from C57Bl/6J mice 21 ± 1 days after infection with 200 *T. spiralis* larvae.

Splenic Cell Populations

Functional or quantitative changes in splenic cell populations induced by infection are possible reasons for the suppression of the antibody response to SRBC observed in parasitized mice. Splenocyte populations from normal and infected mice were compared by differential cell counts, mitogen responsiveness, and relative numbers of T and B lymphocytes in an attempt to detect changes of possible significance in the immune systems of parasitized mice.

Differential cell counts were made of spleen cell suspensions from mice infected for varying lengths of time (Table 9). There was no significant change in total cell numbers at any of the times the spleens were examined. By 20 days after infection, there was a shift in cell populations to large lymphocytes, blastoid cells, and eosinophils with a concommitant reduction in small lymphocytes.

Splenocytes from normal mice or mice infected for 7, 14, or 20 days were cultured in the presence of the T-cell mitogens, phytohemagglutinin (PHA) and Concanavalin A (Con A), and the B-cell mitogen, lipopolysaccharide (LPS). These results are shown in Figs. 6, 7, and 8. The LPS responses from infected mice were essentially normal at all time periods. The responses to the T-cell mitogens were normal seven days after infection, but were suppressed 14 and 20 days after infection.

TABLE 9

SPLENIC CELL POPULATION

CELL TYPES[a]	NORMAL MICE	MICE INFECTED FOR 7 DAYS	14 DAYS	20 DAYS
Cells per spleen ($X10^{-7}$)	8.8 ± 0.6	9.1 ± 0.8	8.8 ± 0.6	8.4 ± 0.5
Small Lymphocytes	79 ± 1%	76 ± 2%	76 ± 1%	60 ± 4%
Large Lymphocytes[b]	16 ± 1%	19 ± 3%	19 ± 2%	24 ± 3%
Macrophages	2 ± 0.3%	3 ± 1%	2 ± 0.5%	3 ± 1%
Eosinophils	0 %	2 ± 1%	3 ± 2%	11 ± 4%

a. Data are means ± sem of two to eight mice per group.

b. Includes blastoid cells.

FIGURE 6. Mitogen responses of splenocytes seven days postinfection. Each point is one experiment; horizontal lines are means of experiments within each group. Data are percent of normal response:

$$\frac{\text{cpm, cells from infected mice (stimulated - background)}}{\text{cpm, cells from normal mice (stimulated - background)}} \times 100$$

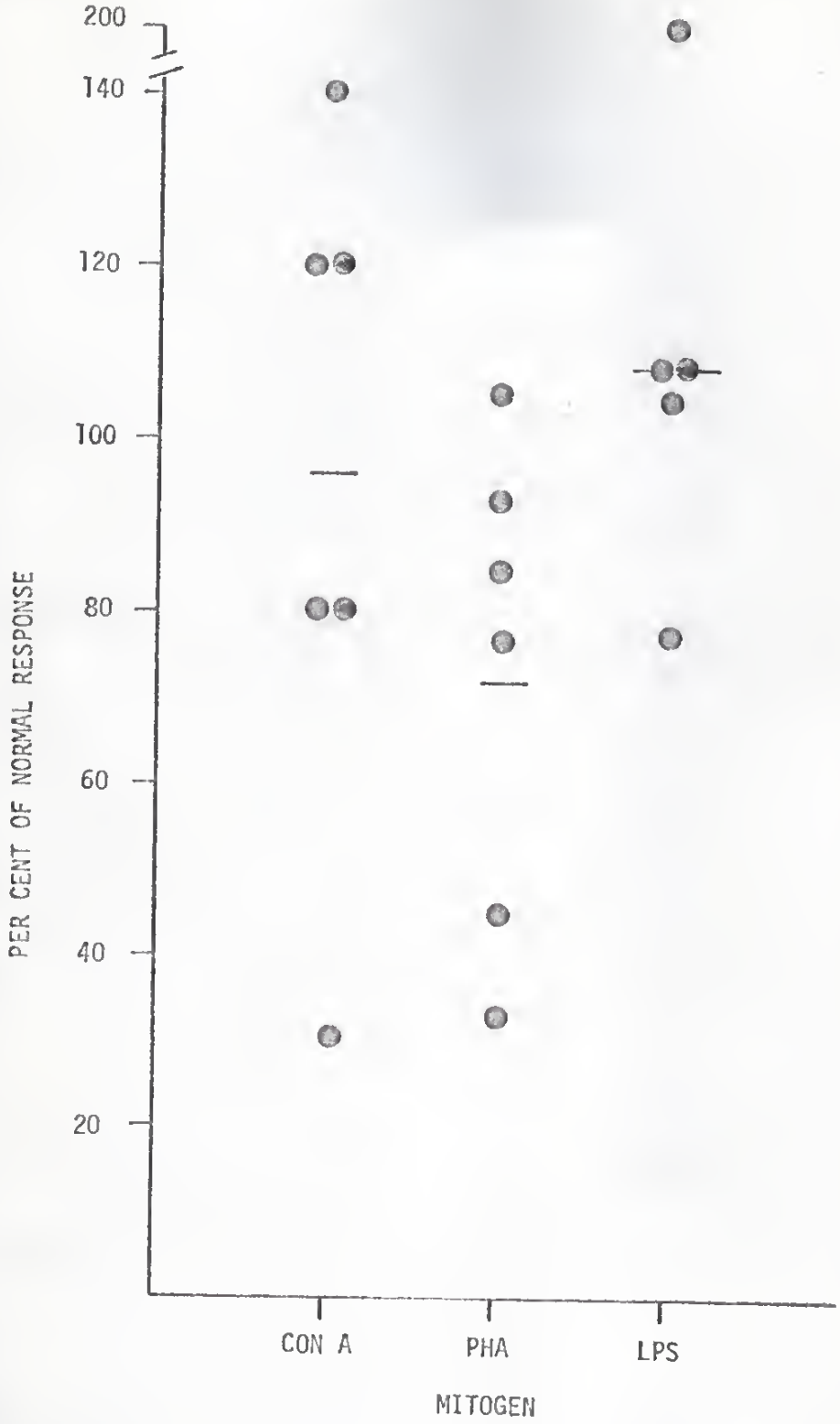

200
140
120
100
80
60
40
20
PER CENT OF NORMAL RESPONSE
CON A
PHA
LPS
MITOGEN

FIGURE 7. Mitogen responses of splenocytes 14 days postinfection. See Figure 6 for explanation of axes. Con A and PHA responses were suppressed significantly.

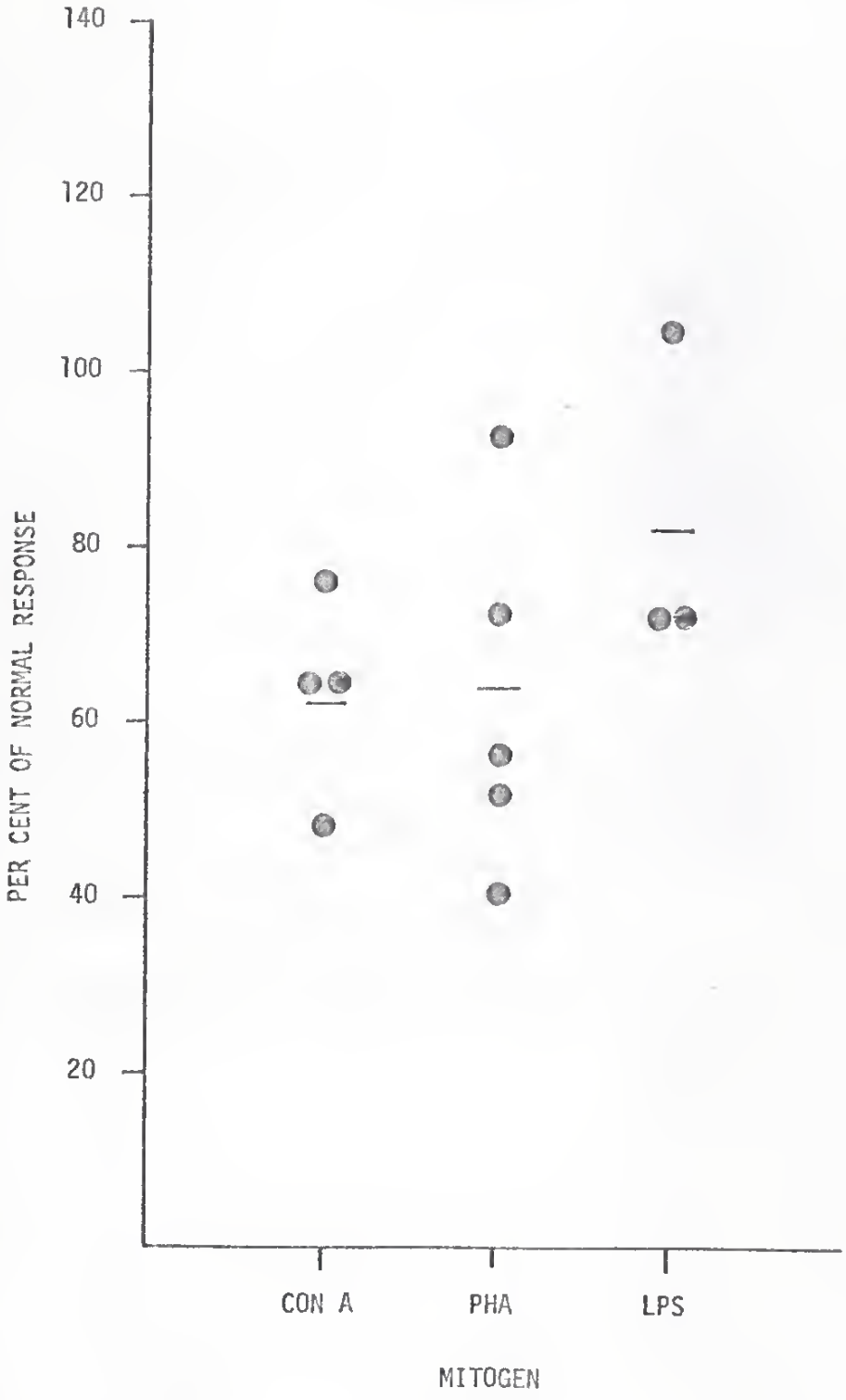

140
120
100
80
60
40
20
PER CENT OF NORMAL RESPONSE
CON A
PHA
LPS
MITOGEN

FIGURE 8. Mitogen responses of splenocytes 20 days postinfection. See Figure 6 for explanation of axes. Con A and PHA responses were suppressed significantly.

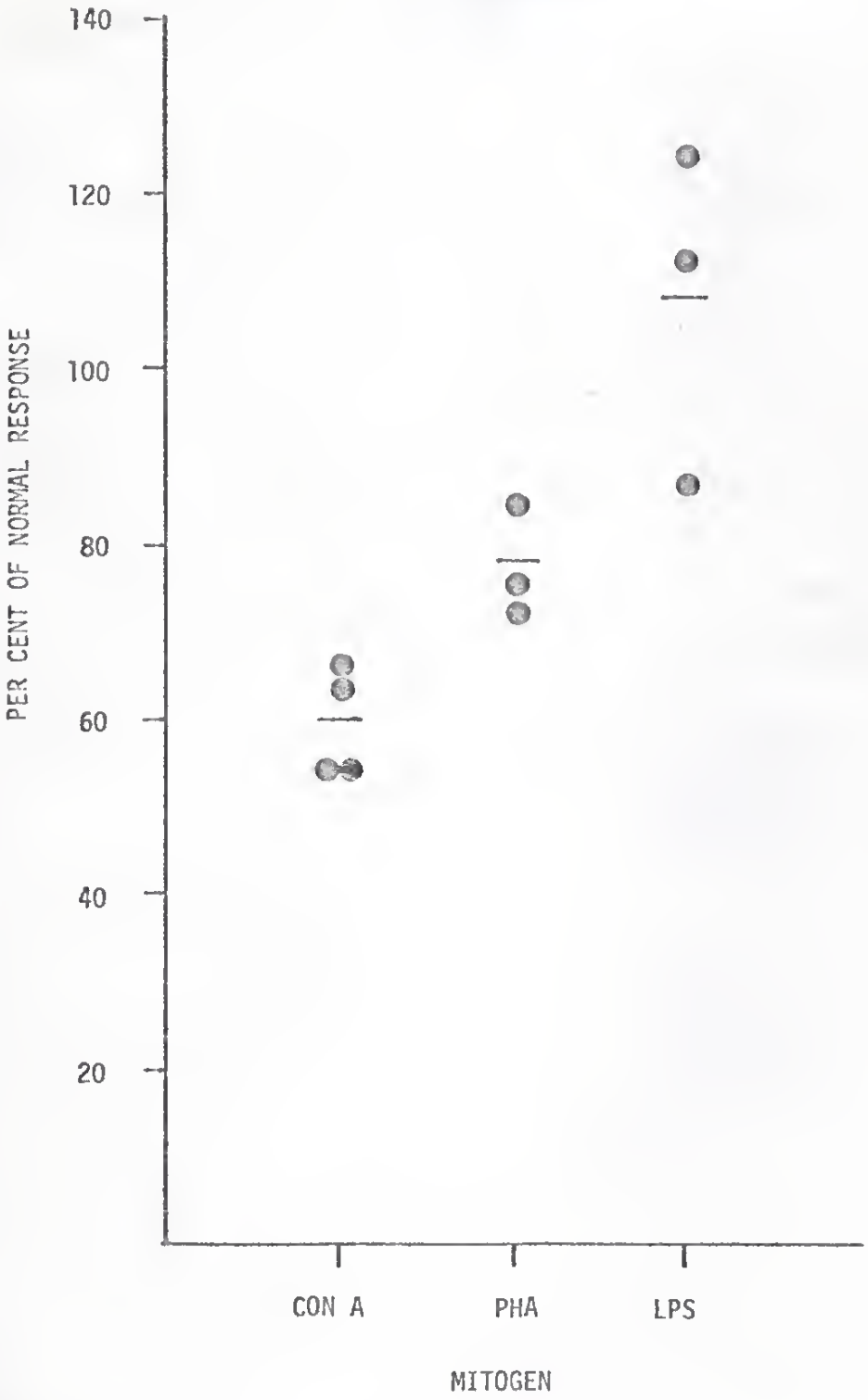

140
120
100
80
60
40
20
PER CENT OF NORMAL RESPONSE
CON A
PHA
LPS
MITOGEN

TABLE 10

SPLENIC LYMPHYCYTE POPULATIONS

MICE (N)[a]	PERCENT CYTOTOXICITY[b]	
	ANTI-THY ANTISERUM	ANTI-IG ANTISERUM
Control (2)	32 ± 1	38 ± 3
Infected (4)	32 ± 1	41 ± 2

a. Infected mice were infected 20 days before assay. Pooled splenocytes from three to six mice. (N)= number of pools tested.

b. Cytotoxicity with a 1:24 dilution of antiserum. Mean ± sem.

The relative proportions of T-cells and B-cells were assayed by anti-Thy-1 and anti-Ig cytotoxicity. These results, shown in Table 10, demonstrate that spleens of normal and 20-day infected mice had the same proportion of T- and B-cells.

Suppressor Cells

Since immunosuppression of the primary antibody response to SRBC could be "active," due to the presence of a suppressive cell population, or "passive," due to alterations in the proportions of cells present or loss of a functional population, the following experiment was done to distinguish between these possibilities. Varying numbers of cells from mice infected for 20 days or from normal mice were added to cultures of 10^7 normal splenocytes. As shown in Fig. 9, both 2.5 and 5 x 10^6 splenocytes from infected mice were able to suppress significantly the number of AFC produced by normal cells, suggesting that a suppressor cell population was present in the spleens of infected mice. Active suppression was confirmed in three other experiments.

To investigate the identity of this suppressor population, splenocytes were treated with anti-thy-1 antiserum plus complement; 2.5 x 10^6 of the remaining viable cells were added to cultures of 10^7 normal splenocytes. The results, shown in Table 11 A, show that treatment with anti-thy antiserum, which selectively kills T-cells, abolished suppression. In another experiment, splenocytes were treated with anti-Ig plus complement, and 1.25 x 10^6 of the remaining viable cells were added to cultures of 10^7 normal cells. As shown in Table 11 B, these few cells from infected mice, if untreated, were not able to suppress the immune responses of normal splenocytes. However, following treatment of these cells with anti-Ig, a procedure which enriches the T-cell population by eliminating mainly B-cells, suppression was significantly increased.

FIGURE 9. Active suppression by splenocytes from infected mice. ○ Responses of 10 million normal splenocytes plus additional normal splenocytes. ● Responses of 10 million normal splenocytes plus splenocytes from 20-day infected mice. ■ Responses of 10 million splenocytes from 20-day infected mice plus additional cells from infected mice. Mean ± sem of four replicate cultures. Both doses of cells from infected mice suppressed significantly the AFC responses of cells from normal mice ($p < 0.05$).

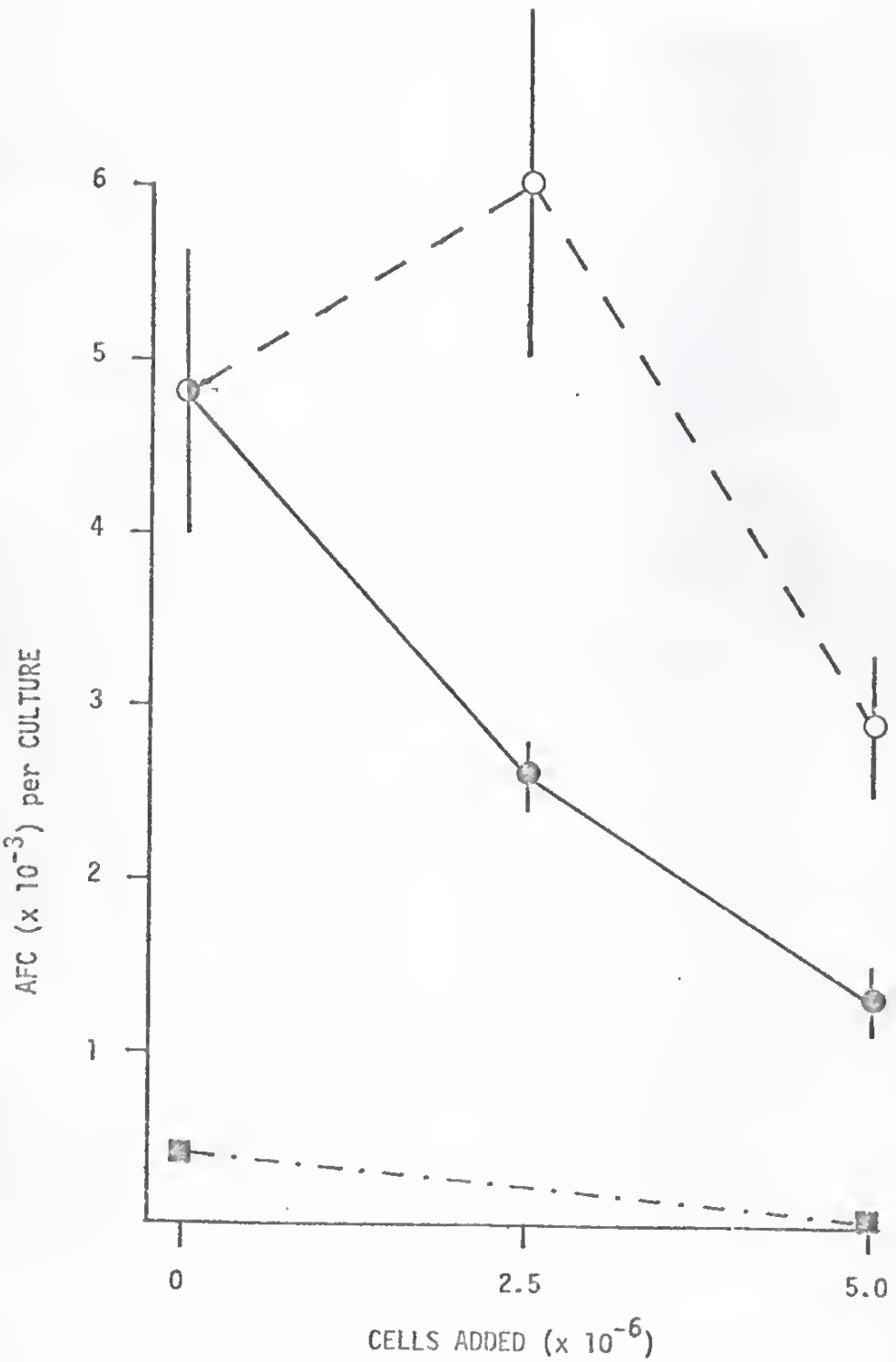

6
5
4
3
2
1
AFC (x 10^-3) per CULTURE
0
2.5
5.0
CELLS ADDED (x 10^-6)

TABLE 11

EFFECT OF ANTI-THY AND ANTI-IG ANTISERA ON THE IMMUNOSUPPRESSIVE ACTIVITY OF SPLENOCYTES FROM INFECTED MICE

EXPERIMENT	TREATMENT OF ADDED CELLS	NO. OF ADDED CELLS	AFC/CULTURE[a] NORMAL MICE[b]	AFC/CULTURE[a] INFECTED MICE[b]
A	None	2.5×10^6	8,600 ± 800	3,240 ± 280[c]
	Anti-thy + C'	2.5×10^6	7,870 ± 480	7,460 ± 1,000
B	None	1.25×10^6	6,575 ± 1,110	5,120 ± 230
	Anti-Ig + C'	1.25×10^6	4,630 ± 1,140	2,630 ± 460[c]

a. Mean ± sem of four replicate cultures.

b. Source of added cells.

c. Significantly lower ($p < 0.05$) than other three values.

Immunosuppressive Factors

In many systems in which suppressor cells have been demonstrated, soluble mediators were found to be important (41, 112, 113). To determine if such a factor might be involved in the *in vitro* suppression demonstrated here, supernatant fluids from cultures of splenocytes containing no SRBC were concentrated and 0.6 ml (three culture-equivalents) was added to cultures of normal splenocytes plus SRBC. As shown in Table 12, addition of supernatants from cultures of splenocytes from 20-day infected mice significantly suppressed the responses of normal cells when the supernatants were added on either Day 0 or Day 2. Although supernatants from normal cultures were suppressive, which had been reported by others (114), supernatant fluids from cultures of cells from infected mice were significantly more suppressive.

In another experiment, concentrated supernatant fluids were heated to 56°C for one hour and 0.6 ml of this heated supernatant was added to cultures of normal splenocytes on Day 0. As shown in Table 13, heating under these conditions did not affect the suppressive activity of culture supernatant fluids. Additionally, selected supernatants (two pools from normal cultures, three pools from cultures of cells from infected mice) were analysed for interferon activity by Dr. George Gifford (Department of Immunology and Medical Microbiology, University of Florida). No interferon activity was detected in any of the supernatants from cultures of splenocytes from infected mice.

To determine if an immunosuppressive factor was demonstrable in the sera of infected mice, varying amounts of pooled sera from normal or 20-day infected mice were added to cultures of 10^7 normal cells on Day 0. The results, shown in Fig. 10, indicate that serum from infected mice was no more suppressive than that of normal mice. In addition, neither

TABLE 12

SUPPRESSION OF DEVELOPMENT OF AFC IN VITRO BY SUPERNATANT FLUIDS FROM CULTURES OF CELLS FROM INFECTED MICE

EXPERIMENT	PERCENT OF NORMAL RESPONSE[a] DAY 0[b]	DAY 2[b]
A	29	44
B	5	43
C	25	Not done

a. Percent of normal response = $\frac{\text{AFC/culture with } \underline{\text{Trichinella}} \text{ supernatant}}{\text{AFC/culture with normal supernatant}} \times 100$

In all cases, suppression by *Trichinella* supernatant was significant, $p < 0.05$.

b. Day on which supernatant was added to culture.

TABLE 13

EFFECT OF HEAT ON SUPERNATANT SUPPRESSION

TREATMENT[a]	CONTROL[b]	INFECTED[b]
None	14,570 ± 2,723	7,850 ± 4,344[d]
56°, one hour	16,350 ± 1,250	6,307 ± 490[c]

a. Concentrated supernatants were heated before addition to cultures on Day 0. Mean ± sem.

b. Source of splenocytes used to generate supernatant fluids.

c. $p < 0.05$.

d. $0.1 > p > 0.05$.

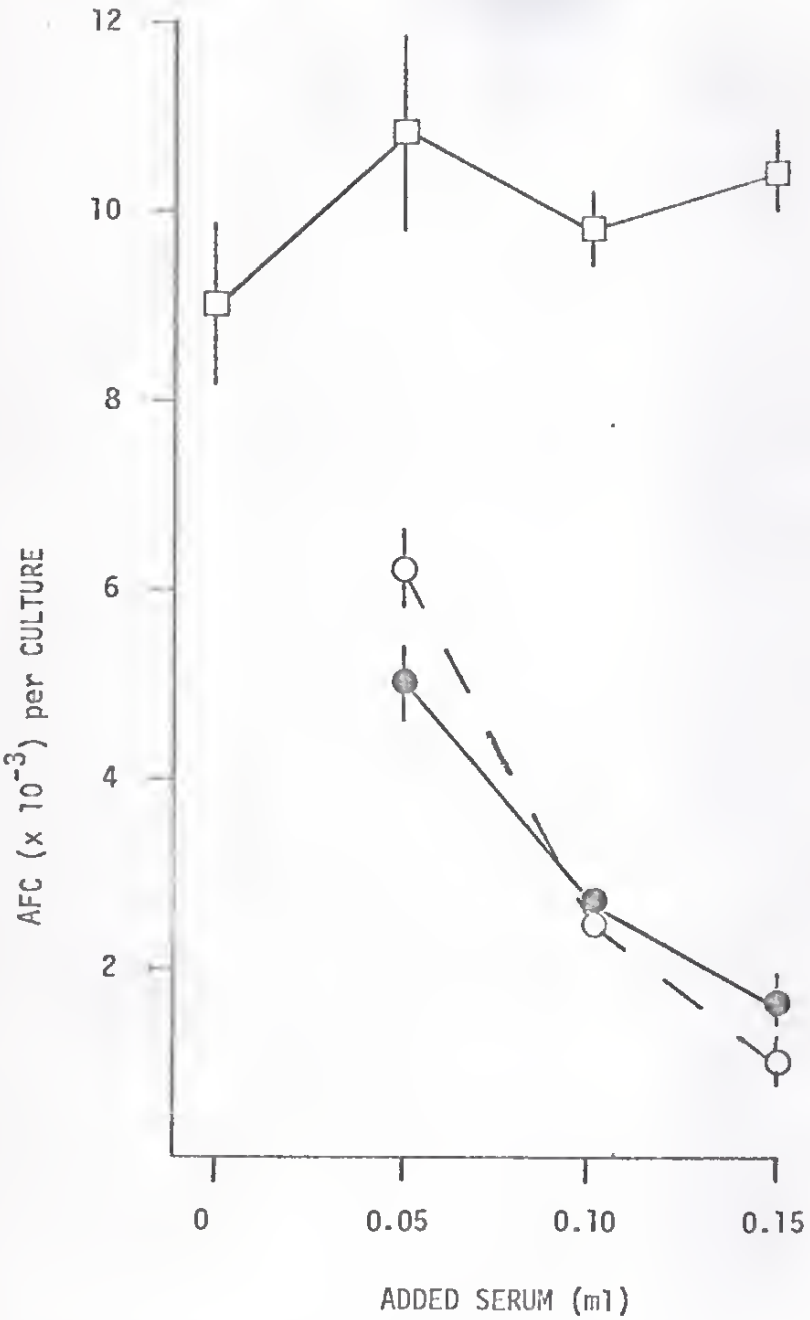


FIGURE 10. Addition of sera to normal spleen cell cultures. ○ Serum from normal mice. ● Serum from 20-day infected mice. □ Fetal calf serum. Mean ± sem of four replicate cultures.

serum pool was cytotoxic. Repeating this experiment using less serum again showed no differences between sera from infected and normal mice.

Immunosuppression by Parasite-derived Products

Other investigators have reported that extracts of Trichinella were suppressive and could contribute to the suppression induced by infections (17). Such activity could be due to the immunogens in the extracts since large amounts of antigen have been shown to induce suppressor cells during in vivo and in vitro immunization (115, 116). Alternately, the helminth products could have direct immunosuppressive effect independent of supressor cell activity.

To evaluate possible immunosuppressive effects, extracts or secretory products from Trichinella larvae known to contain several antigens were added to cultures of normal splenocytes and SRBC. The results (Figs. 11 and 12) show that extracts and secretory products from Trichinella larvae could induce suppression in vitro.

To determine if parasite-derived factors would induce the generation of suppressor cells in vitro, normal splenocytes were incubated for 24 hours with or without 50 µg of Trichinella extract. The cells were then washed three times with Dulbecco's PBS. (Trichinella extract was added to the initial wash of cells incubated without extract.) Varying amounts of these "primed" cells were added to cultures of normal cells plus SRBC. Five days later, the cultures were assayed for AFC. The results, shown in Fig. 13, show that cells incubated with extract were unable to respond normally to SRBC. They did not appear to be actively suppressing the AFC responses of normal cells.

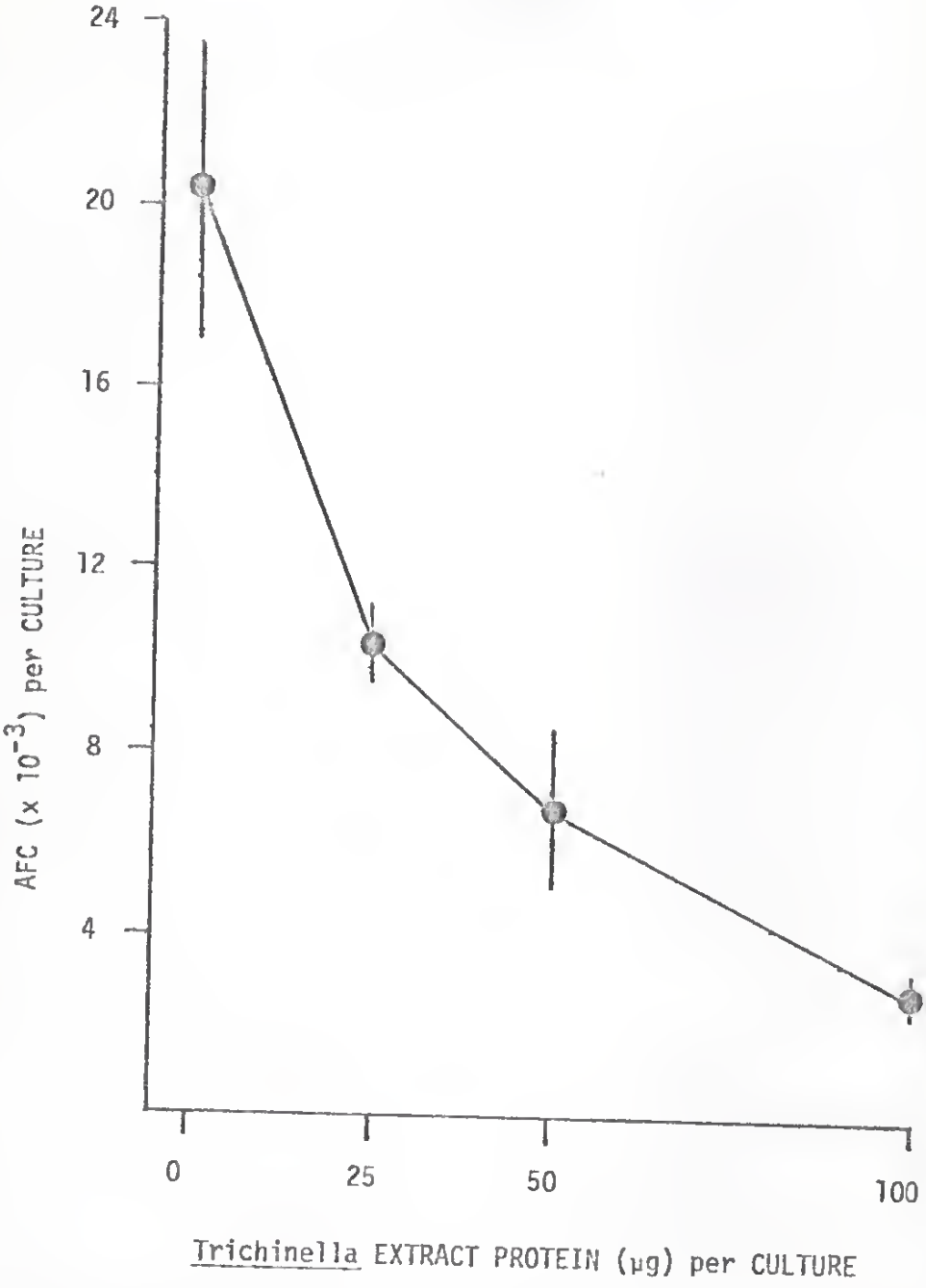


FIGURE 11. Suppression by extracts of Trichinella larvae. Extract was added to cultures of normal splenocytes on Day 0. Mean ± sem of four replicate cultures. All doses of antigen suppressed significantly ($p < 0.01$).

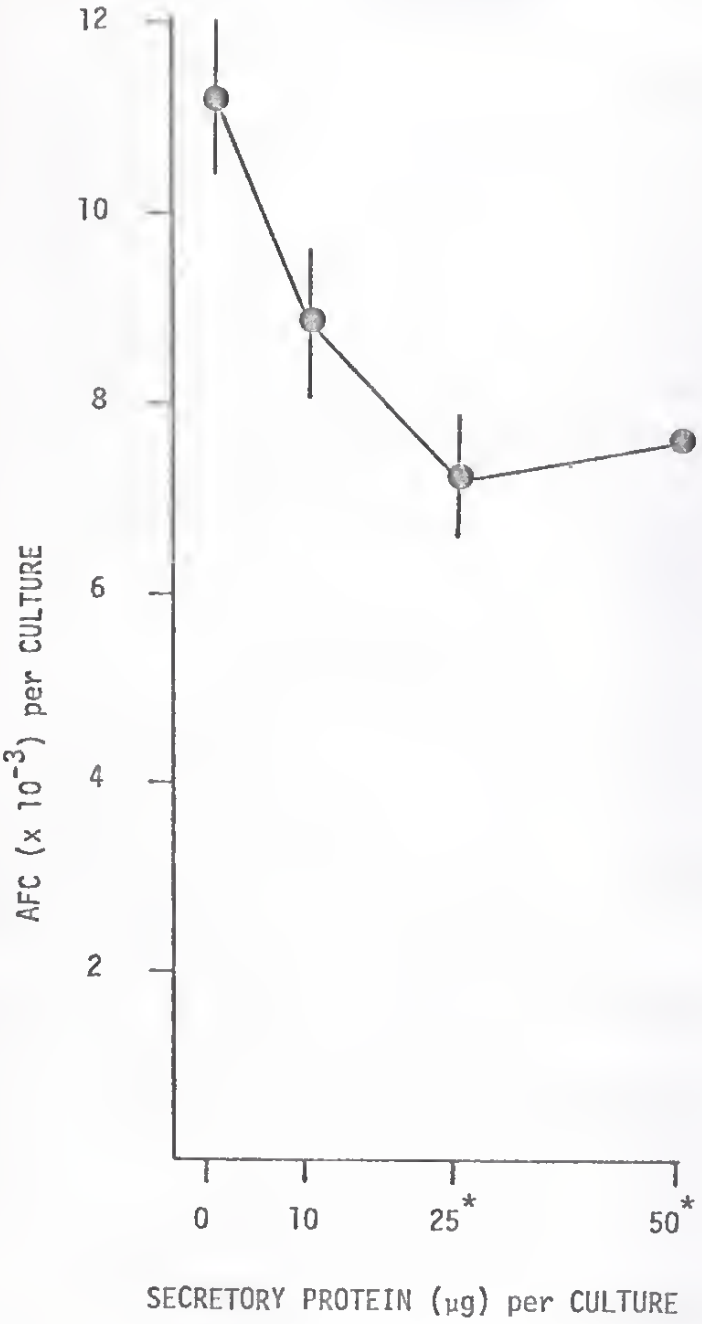


FIGURE 12. Suppression by secretory products from Trichinella larvae. Protein was added to cultures of normal splenocytes on Day 0. Mean ± sem of four replicate cultures. Starred doses suppressed significantly ($p < 0.01$).

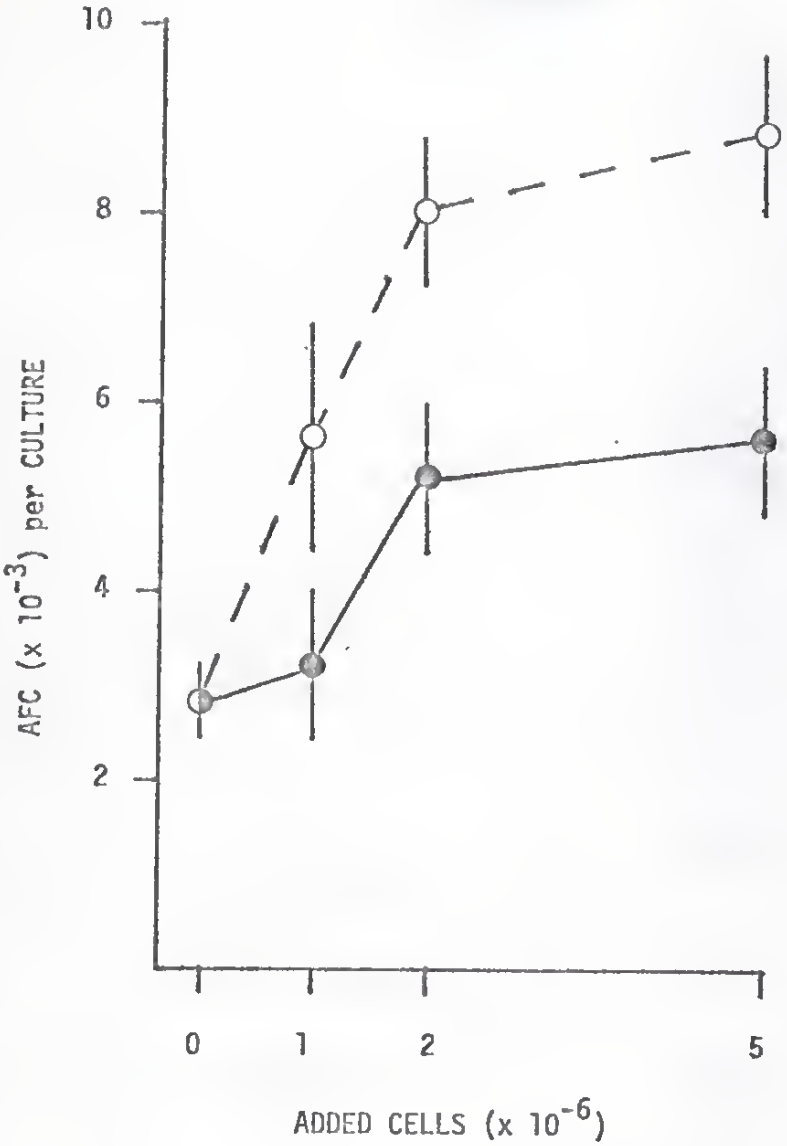


FIGURE 13. Effect of prior incubation with Trichinella extract on development of AFC. Normal splenocytes were incubated with ● or without ○ extract for 24 hours before being added to cultures of ten million normal splenocytes. Mean ± sem of four replicate cultures. Five million cells suppressed significantly ($p < 0.05$).

Mechanisms of Altered Immune Responses by Lymph Node Cells

Lymph node cells from infected mice had suppressed antibody responses to SRBC following *in vivo* immunization and enhanced responses following *in vitro* immunization. The possible reasons for this difference and the mechanisms of *in vivo* suppression were investigated in the experiments described below. In all experiments, unless specifically stated otherwise, C57Bl/6J mice were used 21 ± 1 days after infection with 200 *Trichinella* larvae. In general, axillary and brachial lymph nodes were used for *in vitro* assays, and popliteal lymph nodes were used for *in vivo* assays.

Lymph Node Cell Populations

To determine if there were changes in cell populations that could influence immune responses *in vivo*, cell populations of the axillary and brachial lymph nodes were assessed by differential cell counts, proportions of T- and B-cells, and mitogen responsiveness.

The results of differential cell counts are shown in Table 14. There was at least a three-fold increase in the numbers of cells in the lymph nodes of 20-day infected mice. While there was a decrease in the proportions of small lymphocytes with an increase in large lymphocytes, blastoid cells, and eosinophils, there was an absolute increase in numbers of all cell types.

To determine the types of lymphocytes present, axillary and brachial lymph node cells were assayed for anti-thy-1 antigen and surface immunoglobulin by cytotoxicity assays and for complement receptors by a rosette assay (Table 15). There was a reduction in the proportion of thy-1 bearing lymphocytes (T-cells) and an increase in the proportion of surface Ig- and complement-receptor-bearing cells (B-cells). However, due to the increase in size of the lymph nodes of infected mice, there was an absolute increase in the numbers of all cell types.

TABLE 14

CELL POPULATIONS OF AXILLARY AND BRACHIAL LYMPH NODES

MICE	CELLS PER SET OF LYMPH NODES ($X10^{-6}$)	PERCENT EACH CELL TYPE (NO. PER SET OF LYMPH NODES)			
		SMALL LYMPHOCYTES	LARGE LYMPHOCYTES[a]	MACROPHAGES	EOSINOPHILS
Control[b]	2.6 ± 1.3	90 ± 1%	8 ± 1%	2 ± 0%	0
		($2.3 \pm 0.7 \times 10^6$)	($0.2 \pm 0.1 \times 10^6$)	($0.6 \pm 0.1 \times 10^6$)	
Infected[c]	7.8 ± 0.6	70 ± 2%	24 ± 2%	2 ± 1%	3 ± 1%
		($5.5 \pm 0.6 \times 10^6$)	($1.9 \pm 0.2 \times 10^6$)	($1.7 \pm 0.2 \times 10^6$)	($2.6 \pm 0.5 \times 10^6$)

a. Includes large lymphocytes.

b. Mean ± sem of two pools of cells, nine mice per pool.

c. Mean ± sem of four pools of cells from mice infected for 20 days, four to five mice per pool.

TABLE 15

LYMPHOCYTE POPULATIONS OF AXILLARY AND BRACHIAL LYMPH NODES

MICE	CELLS PER SET OF LYMPH NODES ($X10^{-6}$)	THY-1 BEARING[a]		IG-BEARING[a]		C'3 RECEPTOR BEARING[b]	
		%	NUMBER ($X10^{-6}$)	%	NUMBER ($X10^{-6}$)	%	NUMBER ($X10^{-6}$)
Control[c]	2.0 ± 0.2	72 ± 4%	1.1 ± 0.1	24 ± 4%	0.4 ± 0.1	22 ± 2%	0.6 ± 0.2
Infected[c]	8.4 ± 0.7	34 ± 4%	3.3 ± 0.1	53 ± 2%	3.8 ± 0.2	35 ± 1%	2.7 ± 0.2
Infected as % of control	420%	47%	300%	220%	950%	160%	450%

a. Determined by cytotoxicity assays.

b. Determined by complement-dependent rosette assays.

c. Mean ± sem of two to nine groups, three to nine mice per group. Infected mice had been infected for 20 days.

TABLE 16

MITOGEN RESPONSES OF AXILLARY AND BRACHIAL LYMPH NODE CELLS

	PERCENT OF NORMAL RESPONSE[a]			
	CPM/10^6 CELLS		CPM/SET OF LYMPH NODES	
MITOGEN	EXP. A	EXP. B	EXP. A	EXP. B
PHA	60	63	260	204
Con A	60	61	363	197
LPS	306	223	1,326	718

a. Percent of normal response =

$$\frac{\text{cpm, cells from infected mice (stimulated-background)}}{\text{cpm, cells from normal mice (stimulated-background)}} \times 100$$

Mice had been infected for 20 days at the time of culture.

The mitogen responses of axillary and brachial lymph node cells are shown in Table 16. The proportional responses (responses per 10^6 cells) to the T-cell mitogens were reduced in cultures of cells from mice infected for 20 days, but the total responses were increased. The proportional and total responses to the B-cell mitogen were increased. Additionally, lymph node cells from infected mice had higher background (unstimulated) responses than did those from normal mice, indicating that these cells were already undergoing blastogenesis, probably because of stimulation by parasite antigens.

Taken together, these data show that in the axillary and brachial lymph nodes of mice infected for 20 days there was an absolute increase in all cell types measured, but there was a proportionately greater increase in the B-cell population.

The first of the lymph nodes to be stimulated during Trichinella infection are the mesenteric lymph nodes (117). The number of AFC in these lymph nodes following in vitro immunization were higher than those of normal mice. Immunization of these lymph nodes in vivo was not attempted.

Cell populations of the mesenteric lymph nodes were assessed at various times after infection. The results are presented in Table 17. The mesenteric lymph nodes, like the axillary and brachial lymph nodes, showed a shift to large lymphocytes and blastoid cells at the expense of small lymphocytes, although, due to the increase in size of the lymph nodes, there was an absolute increase in all cell types.

The mitogenic responses of cells from the mesenteric lymph nodes of mice infected for 3, 7, or 14 days are shown in Figs. 14, 15, and 16. Their responses both per million cells and per set of lymph nodes to both T- and B-cell mitogens were higher than the responses of cells

TABLE 17

CELL POPULATIONS OF MESENTERIC LYMPH NODES

CELL TYPE[a]	NORMAL MICE	MICE INFECTED FOR 3 DAYS	7 DAYS	14 DAYS
Cells per set of lymph nodes (10^{-7})	22 ± 0.3	4.1 ± 0.5	4.0 ± 0.4	3.4 ± 0.4
Small lymphocytes	82 ± 2%	85 ± 3%	70 ± 2%	76 ± 2%
Large lymphocytes[b]	15 ± 1%	12 ± 2%	23 ± 2%	19 ± 1%
Macrophages	3 ± 0.3%	2 ± 1%	5 ± 1%	4 ± 1%
Eosinophils	0	1 ± 1%	2 ± 1%	2 ± 1%

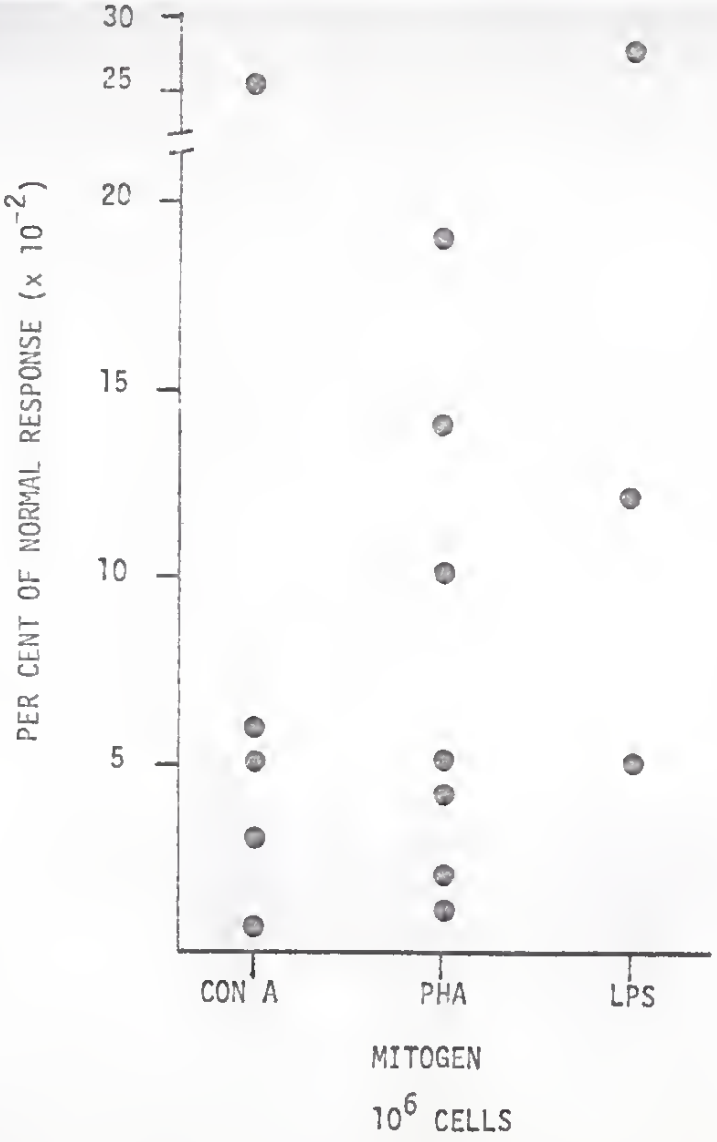
a. Data are means ± sem of three pools of lymph node cells.

b. Also includes blastoid cells.

FIGURE 14. Mitogen responses of mesenteric lymph node cells three days postinfection. See Figure 6 for explanation of axes. Data are presented as responses per million cells and per whole lymph node.

PER CENT OF NORMAL RESPONSE (x 10^{-2})
35
25
15
10
5
CON A
PHA
LPS
MITOGEN
WHOLE LYMPH NODE
PER CENT OF NORMAL RESPONSE (x 10^{-2})
8
6
4
2
CON A
PHA
LPA
MITOGEN
10^6 CELLS

FIGURE 15. Mitogen responses of mesenteric lymph node cells seven days postinfection. See Figure 6 for explanation of axes.


30
25
20
15
10
5
PER CENT OF NORMAL RESPONSE (x 10^{-2})
CON A
PHA
LPS
MITOGEN
10^6 CELLS

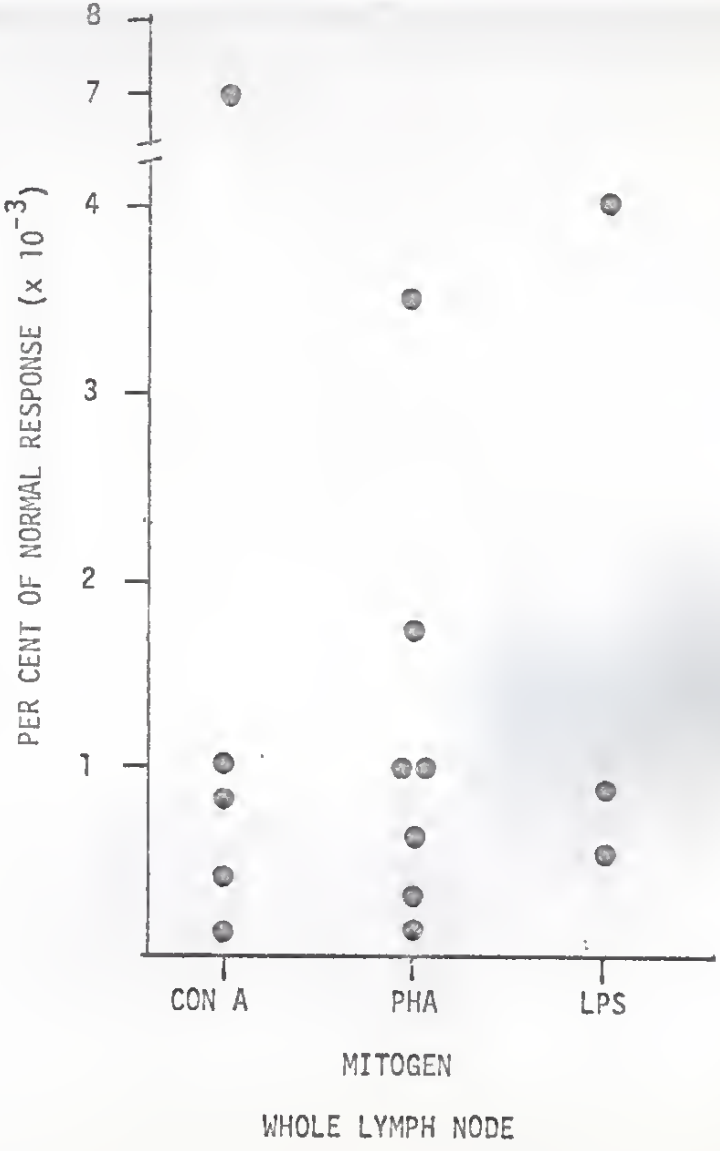
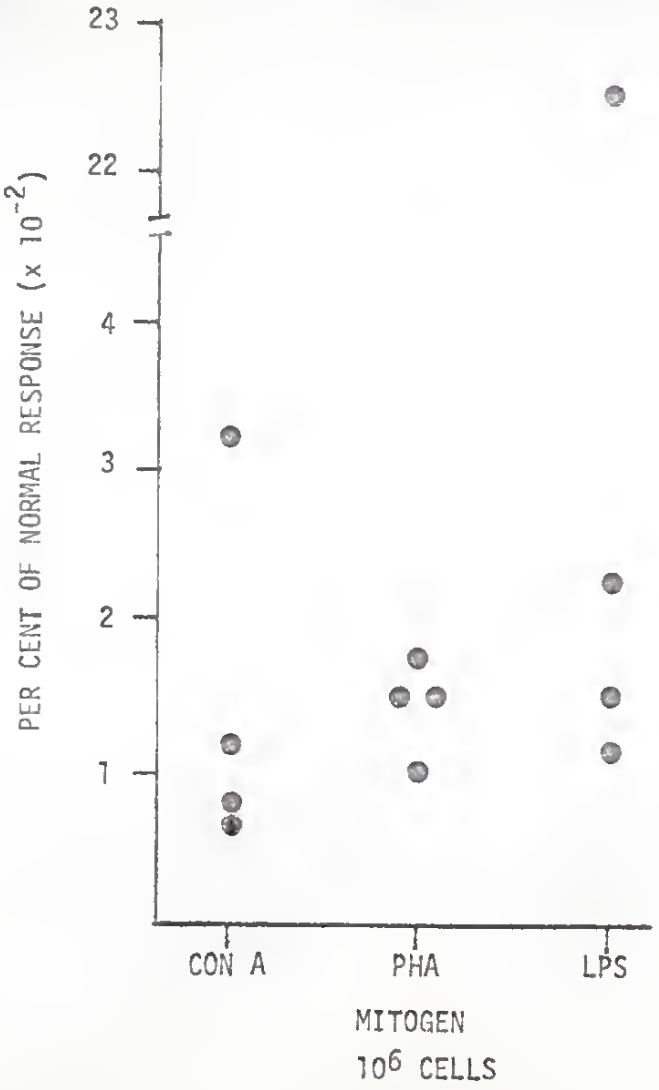
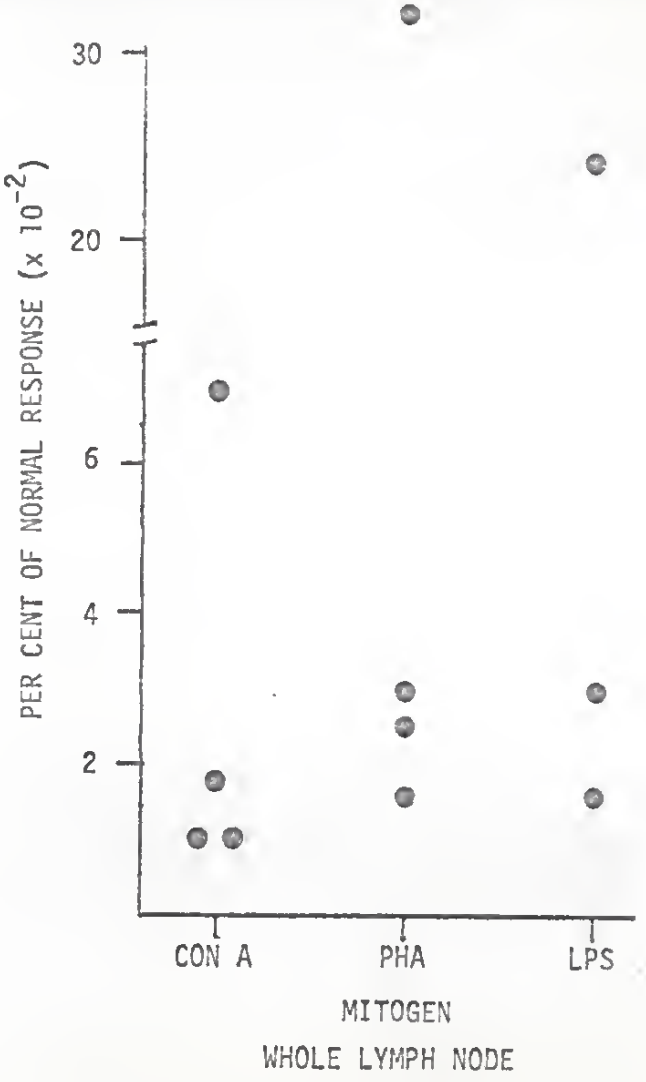

8
7
4
3
2
1
PER CENT OF NORMAL RESPONSE (x 10^{-3})
CON A
PHA
LPS
MITOGEN
WHOLE LYMPH NODE

FIGURE 16. Mitogen responses of mesenteric lymph node cells 14 days postinfection. See Figure 6 for explanation of axes.


23
22
4
3
2
1
PER CENT OF NORMAL RESPONSE (x 10^{-2})
CON A
PHA
LPS
MITOGEN
10^6 CELLS


30
20
6
4
2
PER CENT OF NORMAL RESPONSE (x 10^{-2})
CON A
PHA
LPS
MITOGEN
WHOLE LYMPH NODE

from normal mice. As was found with the axillary and brachial lymph nodes in 20-day infected mice, the mesenteric lymph nodes of infected mice had more cells of all types than did normal lymph nodes.

Lymph node cells from 20-day infected mice gave much higher responses to SRBC following *in vitro* immunization than did normal cells. However, there could have been a suppressor cell population within the lymph node cells of infected mice, without which the responses of these cells would have been even higher. To test this possibility, 2 million axillary and brachial lymph node cells were added to cultures of 10 million normal spleen cells plus SRBC and the cultures were assayed for AFC five days later. As shown in Table 18, these lymph node cells did not suppress AFC responses of normal splenocytes.

Splenic Influence on Lymph Node Cells

Monier (118) has shown that antigenic competition mediated by lymph node cells appears to be dependent on the presence of an intact spleen. The role of the spleen in lymph node suppression was examined in an attempt to explain the contradictory responses to SRBC observed in lymph node cells from infected mice following *in vitro* immunization.

Axillary and brachial lymph node cells from normal or 20-day infected mice were immunized *in vitro* with SRBC in the presence of spleen cells from normal or infected animals. The results, shown in Table 19, indicate that spleen cells from infected mice were able to suppress the *in vitro* responses of lymph node cells from infected mice.

To determine if the spleen might be mediating lymph node suppression *in vivo*, infected or normal mice were splenectomized or sham-operated. Within three hours, the mice were immunized in both hind footpads with 10^8 SRBC. Five days later, their popliteal lymph nodes were assayed for

TABLE 18

EFFECT OF LYMPH NODE CELLS ON SPLENIC AFC INDUCTION FOLLOWING IMMUNIZATION IN VITRO

CELLS IN CULTURE[a]	AFC/CULTURE (MEAN ± sem)	
	EXP. A	EXP. B
10×10^6 NS	470 ± 174	11,020 ± 1,549
12.5×10^6 NS	493 ± 82	11,805 ± 1,154
10×10^6 NS + 2.5×10^6 NLN	593 ± 45	12,000 ± 1,192
10×10^6 NS + 2.5×10^6 TLN	1,245 ± 173	12,655 ± 2,986

a. NS = normal spleen cells; NLN = lymph node cells from normal mice; TLN = lymph node cells from mice infected 20 days previously with Trichinella.

TABLE 19

IN VITRO SUPPRESSION OF LYMPH NODE CELL RESPONSES BY SPLENOCYTES FROM INFECTED MICE

	AFC/CULTURE		
LYMPH NODE CELLS[a]	LYMPH NODE[b]	N-SPLEEN[b]	T-SPLEEN[b]
Normal	100 ± 10	880 ± 67	574 ± 183
Infected	2,625 ± 227	2,455 ± 105	700 ± 87

a. Eight million axillary and brachial lymph node cells from normal or 20 day infected mice were cultured with SRBC and two million cells from lymph nodes (of the same type as the basic culture), normal spleens, or spleens of 20-day infected mice. Data are means ± sem of four replicate cultures.

b. Source of additional cells. N-Spleen = normal spleen; T-Spleen = spleens from mice infected for 20 days.

AFC. The results, presented in Table 20, indicate that splenectomy had no effect on the *in vivo* suppression observed in infected mice. However, there was a reduction in the responses of uninfected, splenectomized mice when compared with uninfected, sham-operated mice, which could have been due to the stress of surgery. Other workers have reported varying degrees of suppression of immune responses following splenectomy which seemed to be related to the time interval between splenectomy and immunization (92, 93). Additionally, suppressor cells, perhaps derived from the spleen, could have been in circulation and been recruited or trapped in the lymph nodes following antigenic challenge.

Consequently, a second experiment was performed. Mice were splenectomized or sham-operated; two weeks later half of them were infected with *Trichinella*. Twenty days after infection, all mice were immunized with SRBC in each hind footpad. Five days later their popliteal lymph nodes were assayed for AFC. The results, shown in Table 21, confirmed that the spleen was not necessary for lymph node suppression, since splenectomized and sham-operated mice infected with the parasite were suppressed equally. However, the spleen probably does have a role in resistance to the parasite, since splenectomized mice had significantly more muscle larvae than did sham-operated mice.

Fate of Injected Antigen

Another hypothesis to explain *in vivo* suppression of the SRBC antibody response is that sufficient antigen does not reach the lymph nodes of infected mice. To test this, 40 µl of a 10% suspension of [^{125}I]-SRBC were injected into the hind footpads of normal and infected mice. On four consecutive days, mice were killed and various organs were assayed for radioactivity. The results are shown in Tables 22 and

TABLE 20

ANTIBODY-FORMING CELLS TO SHEEP ERYTHROCYTES IN POPLITEAL LYMPH NODES OF MICE SPLENECTOMIZED AFTER INFECTION

TREATMENT[a]	MICE[b] (N)	CELLS PER SET OF LYMPH NODES (X 10^{-6})	AFC/10^6 CELLS	AFC/SET OF LYMPH NODES
Splenectomy	Control (7)	3.7 ± 0.4	355 ± 32	1,337 ± 179
	Infected (8)	3.0 ± 0.2[c]	250 ± 43	710 ± 110[d]
Sham-operation	Control (7)	4.3 ± 0.3	745 ± 35	3,243 ± 291
	Infected (7)	3.0 ± 0.3[c]	247 ± 72[d]	751 ± 240[d]
No SRBC	Control (4)	1.2 ± 0.1	0	0
	Infected (4)	1.9 ± 0.1[d]	0	0

a. Mice were splenectomized or sham operated and three hours later injected with 10^8 SRBC into each hind footpad. AFC were assayed five days later. Mean ± sem. No SRBC = includes two sham and two splenectomized mice each group since there was no difference between sham and splectomized mice.

b. (N) = number of mice per group. Infected mice had been infected 20 days before surgery.

c. Infected less than control (borderline, $0.1 > p > 0.05$).

d. Infected less than control, $p < 0.05$.

TABLE 21

ANTIBODY-FORMING CELLS TO SHEEP ERYTHROCYTES IN POPULITEAL LYMPH NODES OF MICE SPLENECTOMIZED BEFORE INFECTION

TREATMENT[a]	MICE[b] (N)	CELLS PER SET OF LYMPH NODES (X 10^{-6})	AFC/10^6 CELLS	AFC/SET OF LYMPH NODES	TOTAL LARVAE/MOUSE
Splenectomy	Control (5)	7.4 ± 0.4	949 ± 133	5,916 ± 77	-
	Infected (7)	5.2 ± 0.4[c]	215 ± 47[c] (23%)	1,041 ± 276[c] (17%)	24,196 ± 3,479[d]
Sham-operation	Control (6)	6.2 ± 0.5	700 ± 129	3,875 ± 906	-
	Infected (7)	5.6 ± 0.5	343 ± 51[c] (49%)	1,515 ± 270[c] (39%)	12,008 ± 1,597[d]

a. Mice were splenectomized or sham-ooperated and two weeks later half the mice were infected with Trichinella. Twenty days after infection, mice were immunized by injecting 10^8 SRBC into each hind footpad. AFC were assayed five days later. Mean ± sem.

b. (N) = Number of mice per group.

c. Infected less than control ($p < 0.05$).

d. Splenectomy greater than sham ($p < 0.01$).

23. There was no difference in the amount of label detected in the popliteal lymph nodes of normal and infected mice. However, infected mice did have less radioactivity in their blood, livers, and kidneys by Day 3 following immunization.

TABLE 22

FATE OF [^{125}I]-SRBC FOLLOWING SUBCUTANEOUS INJECTION: TOTAL COUNTS

DAY[a]	GROUP[b]	ORGAN[c]					
		BLOOD	HIND FEET	POPLITEAL LYMPH NODES	SPLEEN	KIDNEY	LIVER
1	C	10,494 ± 2,870	178,038 ± 7,126	1,844 ± 264	252 ± 67	4,282 ± 611	4,570 ± 618
	T	11,138 ± 1,196	164,539 ± 14,817	1,972 ± 321	295 ± 24	4,040 ± 276	5,036 ± 316
2	C	1,712 ± 238[e]	99,934 ± 5,214	1,565 ± 305	75 ± 12	3,622 ± 247	2,330 ± 169
	T	2,670 ± 195	104,784 ± 5,090	2,190 ± 317	103 ± 7	3,247 ± 104	2,402 ± 178
3	C	206 ± 62[e]	100,831 ± 4,504[e]	1,568 ± 155	86 ± 15	3,696 ± 91[e]	2,429 ± 118[e]
	T	52 ± 10	73,310 ± 4,832	1,042 ± 228	57 ± 5	2,889 ± 194	1,400 ± 141
4[d]	C	71 ± 19	94,310 ± 5,045	1,503 ± 385	49 ± 1[e]	3,673 ± 89[e]	2,329 ± 196[f]
	T	28 ± 12	79,683 ± 12,576	820 ± 206	29 ± 1	2,862 ± 141	1,169 ± 276

a. Mice injected into each hind footpad with 0.04 ml of a 10% [^{125}I]-SRBC solution (10^6 cpm/mouse) on day 0. On the indicated day, groups of four mice were killed, and indicated organs assayed for radioactivity.

b. C = control (uninfected) mice; T = mice infected for 22 days at time of injection.

c. Data are cpm $\pm$ sem of total counts per whole organ.

d. Three mice per group

e. $p < 0.05$.

f. $0.1 > p > 0.05$ (borderline).

TABLE 23

FATE OF [^{125}I]-SRBC FOLLOWING SUBCUTANEOUS INJECTION: SPECIFIC ACTIVITY

DAY[a]	GROUP[b]	ORGAN[c]					
		BLOOD	HIND FEET	POPLITEAL LYMPH NODES	SPLEEN	KIDNEY	LIVER
1	C	6,740 ± 1,705	828 ± 40	103 ± 38	5 ± 1	19 ± 2	6 ± 1
	T	7,718 ± 933	850 ± 89	73 ± 18	6 ± 0.4	18 ± 2	6 ± 0.4
2	C	1,028 ± 147[d]	494 ± 47	81 ± 23[e]	1 ± 0.3	17 ± 2	3 ± 0.2
	T	1,792 ± 150	547 ± 33	177 ± 30	2 ± 0.3	16 ± 1	3 ± 0.2
3	C	1,225 ± 361[e]	520 ± 51	94 ± 24	2 ± 0.4	17 ± 1	3 ± 0.1[d]
	T	360 ± 75	391 ± 33	67 ± 26	1 ± 0.2	14 ± 1	2 ± 0.2
4	C	467 ± 104	434 ± 22	64 ± 23	2 ± 0.4	17 ± 1[d]	2 ± 0.3[e]
	T	210 ± 93	402 ± 50	62 ± 20	1 ± 0.4	12 ± 1	1 ± 0.4

a. Mice were injected into each hind footpad with [^{125}I]-SRBC on day 0 and radioactivity in various organs was measured on indicated day. See Table 22.

b. C = Control (uninfected) mice; T = mice infected for 22 days at the time of injection.

c. Data are cpm $\pm$ sem counts/mg organ weight (or ml blood).

d. $p \leq 0.05$.

e. $0.1 > p > 0.05$ (borderline).

DISCUSSION

Splenic Responses

Several important points concerning spleen-mediated immune responses to unrelated antigens in infected mice have been established by this work. *Trichinella* infection induced a suppression of the primary immune response to sheep erythrocytes following systemic immunization, and this suppression could be demonstrated in cultures of splenocytes immunized *in vitro*. When added to cultures of normal splenocytes, spleen cells from infected mice actively suppressed the *in vitro* antibody response to SRBC, and this suppression was T-cell dependent. Supernatant fluids from cultures of cells from infected mice suppressed the primary *in vitro* antibody responses of normal splenocytes, as did extracts and secretory products from *Trichinella* larvae. However, while the primary antibody response to SRBC was suppressed in infected mice, the delayed hypersensitivity response to SRBC was not, and neither was the antibody response to the T-independent antigen, DNP-Ficoll.

Immunosuppression induced by *Trichinella* has been reported by several investigators, and suppression of the *in vivo* humoral antibody response is also well established. The present work confirmed those findings and provided experimental evidence for the mechanisms responsible for this suppression.

The data presented here are compatible with many of the features of sequential antigen-induced suppression (AIS). Infection with *Trichinella* produces a complex, intense, and persistent antigenic stimulus. Twenty

days after infection, when immunosuppression is demonstrable, infected mice are synthesizing large amounts of antibody to the parasite and their lymphocytes are responsive to Trichinella antigens (5). Several studies have demonstrated AIS in vitro in situations similar to the one described here. When splenocytes from mice immunized with Trichinella were cultured with SRBC in vitro, their antibody responses to SRBC were impaired. This is similar to the demonstration of AIS by Sjöberg and Britton (119) and Pross et al. (120), who showed that the immune response to one antigen following in vitro immunization was suppressed if the splenocytes had previously been immunized in vivo to an unrelated antigen. Additionally, Sjöberg and Britton showed that splenocytes immunized in vivo to one antigen could suppress the antibody responses of normal splenocytes immunized in vitro to a different antigen (119), which parallels the evidence in Fig. 9 for active suppression. Gershon and Konda showed that AIS is dependent on suppressor T-cells (38), and suppressor T-cells were demonstrated in the spleens of Trichinella-infected mice (Table 11). Antigen-induced suppression is observed only if the first antigen is T-dependent (39), and the immune response to Trichinella is T-dependent (7, 8). Finally, Thomas et al. showed that in vitro AIS is mediated by a soluble suppressor factor (41), and a soluble suppressor was demonstrated in cultures of cells from infected mice(Table 12).

Suppressor or regulatory cells capable of inhibiting immune responses are induced under a variety of circumstances and are apparently heterogeneous (121, 122). They seem to be important in the regulation of normal immune responses (123). In AIS, suppressor cells are probably induced during the initial T-dependent immune response (41, 42). Several investigators have shown that excess antigen can lead to the preferential

development of suppressor cells (115, 116). Eardly and Gershon demonstrated that large numbers of antigen specific suppressor cells could induce non-specific suppression *in vitro* (124). Suppressor T-cells have been shown to suppress delayed-type hypersensitivity (125) and the antibody responses to T-independent antigens (126, 127). However, while cells capable of suppressing the primary antibody response to SRBC could be demonstrated in the spleens of infected mice, no suppression of the delayed hypersensitivity response to SRBC was observed (Fig. 4). Furthermore, the antibody responses to DNP-Ficoll were enhanced in infected mice following *in vitro* immunization (Fig. 3) and were either normal or enhanced following *in vivo* immunization (Table 6). Delayed hypersensitivity was assessed in CD-1 mice, and *in vivo* suppression of the antibody response to SRBC has not been confirmed in these mice in this laboratory.

Helper cells and cells mediating delayed hypersensitivity probably belong to different subpopulations of T-cells (128), and suppressor cells to yet another subset (129). Given the functional and antigenic heterogeneity of T-cells, it is reasonable to assume that, for each T-cell function, there is a suppressor cell. Thus, certain T-cells could suppress only T-dependent antibody responses, others only T-independent responses, and still others delayed hypersensitivity or cell-mediated cytotoxicity. In fact, Whisler and Stobo have demonstrated that cells suppressing direct (IgM) plaques are different from those suppressing indirect (IgG) plaques (130). In *Trichinella* infection, the T-dependent immune responses may generate suppressor cells capable of suppressing only helper function in T-dependent antibody responses.

Extracts and secretory products from *Trichinella* larvae suppressed the primary antibody response to SRBC by normal splenocytes (Figs. 11

and 12) without any demonstrable cytotoxicity. These parasite-derived substances could induce the generation of suppressor cells or could act directly on the lymphocytes, suppressing their responses without the intervention of suppressor cells. Incubating normal splenocytes with Trichinella extract for 24 hours apparently did not result in the generation of suppressor cells (Fig. 13). However, much of the material in this Trechinella extract is probably not antigenic, and consequently, the priming dose of antigen may have been insufficient for generating suppressor cells during a short incubation period. Or, the "primed" cells may need additional Trichinella antigen as well as SRBC in culture in order to be suppressive. Primary suppressor cells require the presence of the inducing antigen to exert their influence, but secondary suppressor cells do not (121). However, the cells which had been incubated with Trichinella extract did not seem to respond to SRBC, perhaps due to a direct inhibitory effect of the extract on the lymphocytes.

Faubert reported that only newborn larvae could suppress the primary in vitro immune response to SRBC in a Marbrook culture system (19). However, his results showed, at best, only borderline suppression by newborn larvae and no suppression by adults or muscle larvae. In contrast, the present results showed that secretory products of muscle larvae did suppress significantly the primary antibody response to SRBC (Fig. 12). The difference in results could be due to the different culture systems used. Alternately, since Faubert used living larvae in the inner chamber with the lymphocytes and SRBC in the outer chamber, the amount of antigen reaching the lymphocytes may have been insufficient to cause suppression.

Faubert and Tanner reported that extracts of Trichinella were leukotoxic (17), but the preparations used in the experiments described here had no deomonstrable cytotoxic activity. Additionally, Faubert and Tanner reported that serum from infected mice was suppressive and leukotoxic (17, 20). However, the data in Fig. 10 showed that serum from infected mice was no more suppressive than normal serum, and there was no evidence of cytotoxicity. The data do not exclude a role for humoral factors during in vivo immunosuppression.

While anti-thy-1 and anti-Ig cytotoxicity assays revealed no loss of T-cells from the spleens of infected mice (Table 10), mitogen studies showed that there was a suppression of the blastogenic responses to Con A and PHA, with the reduction of the Con A response greater than the reduction of the PHA response (Fig. 8). Cells that suppress the SRBC response could also be suppressing the mitogen response, since suppressor cells have been shown to suppress mitogen responses (131). Additionally, Stobo and Paul have differentiated T-cells by Con A and PHA responsiveness and correlated this to the density of thy-1 antigen on cell membranes. They delineated a population of cells with relatively low thy-1 density that responded mainly to Con A, and a second subset that responded equally well to Con A and PHA and had high thy-1 density (132). A preferential loss from the spleens of infected mice of the low thy-1, Con A responding cell population might not be detectable by anti-thy-1 cytotoxicity, but would be readily detectable by Con A responsiveness.

Although active immunosuppression induced by Trichinella has been demonstrated to be T-cell dependent in vitro, the role of another cell type such as the macrophage or the eosinophil cannot be excluded. Macrophages and T-lymphocytes have complex regulatory interactions, and

T-dependent, macrophage-mediated immunosuppression has been demonstrated (48). The most notable change in splenocyte populations during Trichinella infection was the increase in eosinophils, whose role in immunosuppression is unknown.

Lymph Node Responses

The antibody responses to sheep erythrocytes by lymph node cells from mice infected with Trichinella were strikingly different depending on whether immunization occurred in vivo or in vitro. In vivo immunization resulted in reduced AFC, while in vitro immunization resulted in increased AFC responses to SRBC when compared to the antibody responses of lymph nodes of uninfected mice. Delayed hypersensitivity to SRBC and contact hypersensitivity to oxazalone were apparently not affected by Trichinella infection.

Few workers have reported induction of primary immune response by lymph node cells in vitro. Pierce showed that lymph node cells responded poorly in vitro unless B-cells and macrophages were added (133). Lymph nodes from infected mice contained significantly more B-cells than normal lymph nodes, both proportionally and absolutely (Table 15). These additional B-cells may account for the ability of lymph node cells from infected mice to respond well to in vitro immunization.

There are several hypotheses that could explain suppression in vivo. First, the spleen plays a role in lymph node-mediated suppression in vivo. Second, antigen injected into the footpads does not reach the draining lymph nodes in sufficient quantity, or the architecture of the lymph node is altered so that the necessary cell interactions do not take place. Third, the lymphocyte trap is ineffective in infected mice. Fourth, infected mice have a "macrophage malfunction," which may

be due to the presence of excess numbers of macrophages or to the presence of activated macrophages. Fifth, antigen-induced suppression occurs *in vivo* due to the presence of a humoral suppressive factor in the microenvironment of the lymph node which is missing or ineffective *in vitro*. Or sixth, suppression in the lymph node requires the continued presence of *Trichinella* antigen.

Gershon *et al.* (134) and Wu and Lance (135) reported that suppressor T-cells localized preferentially in the spleen, and Monier showed that lymph node-mediated antigen-induced suppression is dependent on a functional spleen (118). Immunization *in vitro* of lymph node cells from infected mice could be inhibited by spleen cells from infected mice (Table 19) but the two experiments in which mice were immunized following splenectomy demonstrated that an intact spleen was apparently not necessary for suppression of the *in vivo* response to SRBC by lymph node cells (Tables 20 and 21).

Mice infected for 20 days have a demonstrable inflammatory response in the muscle, induced by encysting larvae (136). If antigen is injected into the footpad, it could be trapped at the site of injection by the phagocytic cells involved in inflammation and not reach the lymph nodes in sufficient quantity to be immunogenic. However, data from the experiment in which labelled SRBC were injected into footpads of normal and infected mice showed that there was no difference in the amount of label reaching the popliteal lymph nodes of normal and infected mice (Table 22). Infected mice did have less label in their blood, kidneys, and livers three days after immunization, which may indicate a more rapid clearing of circulating antigen, perhaps due to activated macrophages. This experiment did not preclude the possibility that antigen did not reach the germinal centers of the lymph nodes, or that the architecture

of the lymph nodes of infected mice was altered so that the optimal cell-cell or cell-antigen interactions did not occur.

Following *in vivo* immunization there is a transient trapping of lymphocytes by lymphoid organs (94). Antigen-sensitive cells within the spleen or lymph nodes recruit other cells from the peripheral circulation (137). Lymphocyte trapping is dependent on both T-cells (138) and macrophages (139), and is apparently necessary for an optimal *in vivo* immune response (94). Mongini and Rosenberg have reported suppression of the primary *in vivo* response to SRBC by lactic dehydrogenase virus, a suppression which was probably due to a defect in the lymphocyte trap caused by viral-induced macrophage malfunction (140). Impairment in lymphocyte trapping caused by either T-cell or macrophages during *Trichinella* infection could result in reduced immune responses to SRBC *in vivo*.

While the immune response to SRBC requires the interaction of macrophages, T-cells, and B-cells, the nature of this interaction is still not understood (see review 141). Presumably macrophages are involved in presentation of antigen to lymphocytes. Several groups of investigators have showed that macrophage-bound antigen is considerably more antigenic than soluble antigen (141, 142). Lipsky and Rosenthal observed that macrophages bind lymphocytes reversibly (143). They hypothesized a macrophage-associated-antigen initiation of the immune response which proceeds by an initial antigen independent binding of lymphocytes to macrophages. This is followed by antigen-dependent binding and cell division of those lymphocytes bearing receptors for the appropriate antigen (144). During *Trichinella* infection, macrophages could be processing mainly *Trichinella* antigens. Therefore, lymphocytes carrying receptors specific for the various helminth antigens would be

preferentially bound by the macrophages. Subsequently, if a second antigen, namely SRBC, were introduced into this environment, lymphocytes specific for SRBC antigens might be at a competitive disadvantage for binding to macrophages.

One group of workers has reported that macrophages from malaria-infected mice were better at phagocytosis of red blood cells than normal macrophages, but if these macrophages were transferred into normal mice, they could not initiate an immune response to red cells as well as normal macrophages (discussed in 75). Perkens and Makinodan showed that injection of SRBC into the peritoneal cavities of mice stimulated by glycogen resulted in a lower immune response to the sheep cells than if the SRBC were injected into normal mice. From this and other *in vitro* experiments on rates of phagocytosis, they concluded that macrophages actively engaged in phagocytosis do not initiate immune responses as well as unactivated macrophages (145). Cypess showed that, during *Trichinella* infection, macrophages were more phagocytic than normal macrophages (21) and consequently may have been impaired in their ability to initiate immune responses. The more rapid clearance of radio-labelled antigen from the blood of infected mice (Table 22) and the higher background ear swelling following oxazalone challenge (Table 8) may be due to the presence of activated macrophages.

If the *in vivo* immunosuppression were due to a macrophage "defect" suppression would probably not be observed *in vitro*. Lemke and Opitz showed that 2-mercaptoethanol could substitute for macrophage function during *in vitro* immunization. Although one can never be certain that all macrophages have been removed, Lemke and Opitz had so few macrophages remaining in their preparations that attempts to induce an immune response to SRBC in the absence of mercaptoethanol were unsuccessful

(146). The medium used in the culture system for these studies contained 2-mercaptoethanol.

Antigen-induced suppression cannot be ruled out. Frequently, workers studying AIS *in vitro* have had to add both antigens to cultures in order to demonstrate AIS, even though the animals had previously been immunized *in vivo* to one of the antigens (33). Unlike the spleen, which is stimulated by *Trichinella* antigens beginning early in infection (5, 117), the lymph nodes are not antigen sensitive until nearly the third week of infection (117). While secondary suppressor cells do not require the continued presence of antigen to exert their influence *in vitro*, primary suppressor cells do (121). Consequently, although no suppressor cells could be demonstrated in the lymph nodes of infected mice, they could have been present and inactive needing the addition of *Trichinella* antigen to suppress normal immune responses.

Waterston described suppression of the primary *in vivo* response to SRBC in mice previously immunized with pig erythrocytes. However, when he removed splenocytes from mice immunized with pig erythrocytes and cultured them with SRBC, these cells had increased responses to SRBC. He concluded that the *in vivo* environment contained humoral suppressors which he assumed were antibodies arising from cross-reactivity between sheep cells and pig cells (147). Other workers have also observed loss of AIS when lymphocytes were immunized *in vitro* and have suggested a role for humoral factors (33). While *Trichinella* and SRBC do share at least Forssman antigen (148), at no time were increased antibody responses to SRBC observed, either in HA titers or in AFC responses, in infected mice not immunized with SRBC. However, there may be some factor present in the microenvironment of the lymph node that is missing from the *in vitro* environment.

Conclusions

Suppression of the primary antibody response to sheep erythrocytes was demonstrated in mice infected with Trichinella following both intraperitoneal and subcutaneous injection of SRBC. Suppression could also be demonstrated in splenocytes from infected mice immunized in vitro. This in vitro suppression was mediated by suppressor cells. Supernatant fluids of cultures of splenocytes could suppress the AFC responses of normal mice. This is consistent with the hypothesis that the suppressor cells act by releasing soluble mediators. Since neither delayed-hypersensitivity nor antibody responses to a T-independent antigen were reduced, the suppressor cells appeared to be cells that inhibit only T-dependent helper function. However, while these cells were probably important in suppressing the in vitro immune response to SRBC, there is no proof that these cells are active or important in vivo.

The responses of the lymph nodes of infected mice are different from the splenic responses. Lymph node cells from infected mice had suppressed antibody responses following in vivo immunization but enhanced responses following in vitro immunization. While the increased response in vitro may be due to increased numbers of B-cells, the suppressed responses in vivo are not easily explained. The spleen does not appear to be necessary for suppression, and antigen apparently reaches the lymph nodes equally well in normal and infected mice. Other possibilities which remain to be tested have been discussed.

The work presented here suggests that antigen-induced suppression dependent on suppressor cells might be an important mechanism of parasite-

induced immunosuppression in this model system. Infections with other parasites often induce T-dependent immune responses in the host; one result may be the generation of suppressor T-cells. The role of AIS and suppressor cells in these infections needs to be investigated.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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